IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: Davis, Minh Tam B.
David Philip LANE	)	Art Unit: 1642
Application Serial No. 09/403,440	)	Confirmation No: 7276
Filed: January 19, 2000	)	Attorney's Docket No. 39749-0001 APC
For: **MATERIALS AND METHODS RELATING TO INHIBITING THE INTERACTION OF P53 AND MDM2**	)	**Customer No. 77845**

FILED VIA EFS ON MARCH 10, 2009

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 32613-1450

Dear Sir:

This Appeal Brief, filed in connection with the above captioned patent application, is responsive to the Final Office Action mailed on May 22, 2008. A Notice of Appeal was filed on October 17, 2008. This Brief is timely filed requesting a three-month extension of time with necessary fees. Appellants hereby appeal to the Board of Patent Appeals and Interferences from the final rejection in this case.

The following constitutes the Appellants' Brief on Appeal.

I. REAL PARTY IN INTEREST

The real party in interest is University of Dundee, Dundee, DD1 4HN, United Kingdom, by an assignment recorded on January 19, 2000, at Reel 010552 and Frame 0229.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences, known to Appellant, the Appellant's legal representative, or assignee which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1, 2 and 8 are in this application.

Claims 3-7 and 9-27 have been canceled.

Claims 1, 2 and 8 stand rejected and Appellant appeals the rejection of these claims.

IV. STATUS OF AMENDMENTS

No claim amendments were submitted after the final rejection mailed on May 22, 2008, which is appealed herein. All prior amendments were entered.

A copy of the rejected claims in the present Appeal is provided in the Claims Appendix.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the appealed claims concerns *in vitro* disruption of the binding of the tumor suppressor protein p53 and the oncogene mdm2 in cancer cells that do not overexpress mdm2.

Claim 1 is drawn to an *in vitro* method for disrupting the binding of p53 and p53-binding protein mdm2 in a population of cancer cells in which the p53-binding protein mdm2 is not overexpressed, comprising administering to the cells a peptide, less than 25 amino acids in length, and comprising SEQ ID NO: 3.

Claim 2, which depends from Claim 1, specifies that p53 is activated for DNA specific binding and transcription.

Claim 8, which depends from Claim 1, specifies that the peptide has the property of competing with the p53-binding protein mdm2 for binding p53, but does not inhibit DNA specific binding property of p53.

Both p53 and mdm2 were known in the art at the priority date of the present application. The sequence of mdm2 was disclosed in WO 93/20238 and the domains of p53 responsible for binding mdm2 were disclosed in WO 93/20238 and WO 96/02642 (see, page 1, line 14 – page 2, line 21 of the specification). The invention is based on the experimental finding that mdm2 binds p53 in cancer cells in which mdm2 is not overexpressed, and that inhibiting the binding of mdm2 to p53 in such cells can be used to activate p53 function and thus yield tumor suppression (see, page 2, line 34 – page 3, line 5 of the specification).

The specification discloses experiments in which plasmids encoding highly potent peptide inhibitors of the interaction of mdm2 and p53 were expressed in *E. coli* host cells as peptide aptamers on the surface of bacterial thioredoxin. The peptide inhibitors include Thioredoxin Insert Protein TIP 12/1 (SEQ ID NO: 3) (page 3, lines 6-9 and page 20, line 1 – page 21, line 14 of the specification, and Figure 1). Following purification, the ability of the peptide aptamers, including TIP 12/1, to inhibit the p53-mdm2 interaction was confirmed in ELISA assays, in which TIP 12/1 exhibited strong enough inhibitory potential to compete against endogenous levels of wild-type p53 in tumor cells for binding to mdm2 (page 21, lines 16-24; page 25, lines 8 – 35 and Table 1).

The peptide aptamers, including TIP 12/1, were cloned into the vector pcDNA3 (Promega), and microinjected into a rat thyroid epithelial cell line VRn.6, which was known to express wild-type p53 and overexpress mdm2 at the protein level, where the ability of the aptamers, including TIP 12/1, to interrupt the p53-mdm2 interaction was demonstrated (page 21, line 26 – page 23, line 36; page 26, line 2 – page 27, line 1).

Microinjection experiments were then carried out in the mouse prostate-derived cell line T22, which normally contains very low levels of p53 and mdm2 (page 27, lines 2 – 8). These experiments demonstrated the ability of the tested aptamers, including TIP 12/1, to inhibit p53-mdmd2 interaction in T22 cells, which do not overexpress dmd2 (page 27, lines 12-24).

Subsequently, cells of a human osteosarcoma cell line OSA, known to have highly elevated mdm2 levels due to gene amplification, another osteosarcoma cell line U2-OS, having elevated levels of mdm2-mRNA without gene amplification for mdm2, and the breast cancer cell line MCF-7, known to have low p53 expression levels and no reported mdm2 elevation, were transiently transfected with p53-responsive reporter plasmids and TIP 12/1 (page 22, line 34 – page 23, line 19; and page 27, lin2 32 – page 28, line 8). The results show that TIP 12/1 was able to induce p53-dependent transcriptional activation even in cells with undetectable levels of mdm2 (MCF-7 cells and U2-OS cells) (page 29, lines 8-26 and Figure 3).

Further experiments disclosed in the specification demonstrated that disruption of the interaction between p53 and mdm2 with peptide inhibitors, such as TIP 12/1, releases transcriptionally active p53 in tumor cells even when the cells not only do not overexpress mdm2 rather have hardly detectable mdm2 levels (page 29, line 28 – page 30, line 3; page 32, line 34 – page 34, line 2, and Figures 3 and 4).

The combined results of these experiments demonstrated that inhibiting the binding of p53 and mdm2 in cells that do not overexpress mdm2 can be used to activate the function of tumor suppressor protein p53.

The use of peptides less than 25 amino acids in length to disrupt the binding of p53 and mdm2, as claimed in Claim 1, is specifically disclosed at page 2, line 34 – page 3, line 5; page 6, lines 25 to 32; and page 19, line 36 – page 35, end of Table 1 of the specification. The definition of "cells that do not overexpress mdm2" is provided at page 6, lines 25-32.

Specific support for the embodiment when p53 is activated for DNA specific binding and transcription, as claimed in Claim 2, is, for example, at page 6, lines 22-24; page 27, line 25 – 31, and throughout the experimental section of the specification.

Specific support for the embodiment when the peptide administered has the property of competing with the p53-binding protein mdm2 for binding p53, but does not inhibit DNA specific binding property of p53, as claimed in claim 8, is at least at page 6, line 35 – page 8, line 12 and throughout the experimental section of the specification.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The rejection of Claims 1, 2, and 8 under 35 U.S.C. §103 as allegedly being unpatentable over Bottger *et al.*, 1996 (Oncogene, 13:2141-2147), in view of McCann AH *et al.*, (British J Cancer, 71(5):981-5), and further in view of Lee JM *et al.*, 1995 (Cancer and Metastasis Review, 14(2):149-161).

VII. ARGUMENTS

Summary of the Arguments:

Appellant submits that a *prima facie* case of obviousness has not been established. The documents cited in support of the rejection under 35 U.S.C. §103 do not properly represent the state of the art at the priority date of this application, and the rejection itself is based on improper hindsight reconstruction of the claimed invention. Furthermore, the Examiner misrepresented the teaching of the secondary reference, McCann AH *et al.*, 1995 (British J Cancer, 71(5):981-5), and failed to give proper weight and consideration to two expert Declarations under 37 C.F.R. §1.132 by Professor Karen Vousden.

It is submitted that when the issue of obviousness is assessed using the proper legal standard, considering the totality of evidence and arguments of record, the Board should come to the conclusion that the rejection of Claims 1, 2 and 8 is legally and scientifically incorrect and should be overturned.

These arguments are all discussed in greater detail below.

The Rejections

According to the Final Office Action mailed on May 22, 2008, which is appealed herein, Claims 1-2, and 8 are rejected under 35 U.S.C. §103 as allegedly being unpatentable over Bottger *et al.*, 1996 (Oncogene, 13:2141-2147), in view of McCann AH *et al.*, 1995 (British J Cancer, 71(5):981-5), and further in view of Lee JM *et al.*, 1995 (Cancer and Metastasis Review, 14(2):149-161) "for reasons already of record in paper of 09/11/07." The Office Action mailed on September 11, 2007 in turn maintains the same rejection "for reasons already of record in paper of 12/13/06," which is an Advisory Action, maintaining the rejection "for reasons already of record in paper of 07/18/06."

Based on the combined contents of these Office Actions, the Examiner's position appears to be that

Bottger *et al.* teaches:

(i) the consensus sequence PXFXDYWXXL contained in the 12 amino acid peptide MPRFMDYWEGLN (Table 1 on page 2142), which is the same as the peptide MPRFMDYWEGLN of the claimed invention, a 12 amino acid fragment of the 19 amino acid peptide of SEQ ID NO: 3;

(ii) that this 12 amino acid peptide is superior over the wild-type hdm2 binding site of p53 (QETFSDLWKLLP) in its ability to inhibit the binding wild-type p53 and mdm2;

(iii) that the N-terminal region of p53 is important for its interaction with mdm2;

(iv) that the amino acids proline and tyrosine from peptide 12/1 are additional binding points for hdm2, for improved stability, and for better conformational fit into the dm2 binding pocked of p53 to displace the binding of p53 to hdm2;

(v) that the oncogene mdm2 and its human homologue hdm2 bind to p53 and inactivate p53;

(vi) that the mdm2-p53 interaction is a much pursued target for the development of anti-cancer drugs; and

(vii) the 12 amino acid peptide MPRFMDYWEGLN represents a clear route towards the design of small synthetic molecules that will restore p53 function in human tumors.

The Examiner acknowledges that Bottger *et al.* does not teach an *in vitro* method for disrupting the binding of p53 and mdm2 in a population of cancer cells in which mdm2 is not overexpressed comprising administering a peptide, less than 25 amino acids in length, and comprising SEQ ID NO: 3, as claimed in Claim 1 of the present application.

McCann *et al.* is relied on for teaching expression of mdm2 in breast carcinoma and its association with low level of p53, and for allegedly teaching that mdm2 amplification only occurs at a low frequency in breast cancer, as compared to non-epithelial tumors.

Lee JM is relied on for allegedly teaching that p53 could induce apoptosis and cell cycle arrest, and that loss of p53 function causes increased resistance to chemotherapeutic agents. Lee JM was further cited for its alleged teaching that p53 functions as a transcriptional factor, via binding to specific DNA.

From this, the Examiner concludes that it would have been *prima facie* obvious to make a peptide from the mdm2 binding site of p53, wherein the polypeptide comprises the 12 amino acid peptide M**P**R**F**M**D****Y**WEGLN taught by Bottger *et al.*, and where the peptide is of larger size than the 12 amino acid peptide taught by Bottger *et al.* to increase stability, because additional amino acids surrounding the peptide of Bottger *et al* are part of the mdm2 binding site of p53 and thus would be expected to increase stability.

The Examiner further asserts that it would have been obvious to use the peptides of the present invention to target cancer cells that express p53 and mdm2 but do not overexpress mdm2, such as breast cancer cells taught by McCann *et al.*, because loss of p53 function is correlated with increased resistance to chemotherapeutic agents, as taught by Lee *et al.*, and because in cancers which do not overexpress mdm2, such as breast cancer cells, the protein expression of mdm2 is significantly associated with low level of p53, as taught by McCann *et al.*

According to the rejection, a reasonable expectation of success is provided because of the improved inhibitory activity of the consensus sequence P**X**F**X**D**Y**W**XXL** taught by Bottger *et al.*, and because hdm2 binding to p53 has been known to inactivate p53 function, as taught by Bottger *et al.*

With regard to Claim 2, the Examiner notes that one would have excepted that the peptide of the present invention does not inhibit the DNA specific binding property of p53m because the peptide taught by the combined art would disrupt the binding of p53 to mdm2 binding only at the specific p53 binding site of mdm2, as taught by Bottger *et al.*, which is different from the DNA binding site of p53.

With regard to Claim 8, the Examiner adds that one would have expected that p53 is activated for DNA specific binding and transcription, because the activity of p53 is to function as a transcriptional factor, via binding to specific DNA, as taught by Lee *et al.* and Bottger *et al.*

Response to the Rejections

1. *The legal standard*

35 U.S.C. §103 "forbids issuance of a patent when 'the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.'" *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1734, 82 USPQ2d 1385, 1391 (2007).

The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art, (2) any differences between the claimed subject matter and the prior art, and (3) the level of skill in the art. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966). *See also KSR*, 127 S.Ct. at 1734, 82 USPQ2d at 1391. ("While the sequence of these questions might be reordered in any particular case, the [*Graham*] factors continue to define the inquiry that controls."). The Court in *Graham* further noted that evidence of secondary considerations, such as commercial success, long felt but unsolved needs, failure of others, etc., "might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." 383 U.S. at 18, 148 USPQ at 467.

While in *KSR*, the Supreme Court rejected a rigid application of the teaching, motivation, suggestion (TMS) test, an important safeguard against the hindsight bias in obviousness determination based on multiple references, replacing it with a flexible, "common sense" approach, it remains clear that "hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention," (*Ecolochem*, 227 F.3d at 1371 (quoting *In re* Fine, 837 F.2d 1071, 1075 (1988)) is abhorrent to obviousness analysis. Similarly, the Examiner may not use the invention as a blueprint for linking together pieces of prior art in order to find the invention obvious. See, *e.g., Grain Processing Corp. v.American Maize-Products Co.*, 840 F.2d 902, 907, 5 USPQ2d 1788,1792 (Fed. Cir. 1988). The Federal Circuit has also cautioned against focusing on the obviousness of the differences between the claimed invention and the prior art rather than on the obviousness of the claimed invention as a whole as §103 requires. See, *e.g., Hybritech Inc. v. Monoclonal Antibodies,Inc.*, 802 F.2d 1367, 1383,

231 USPQ 81, 93 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987). "It is difficult but necessary that the decision maker forget what he or she has been taught . . . about the claimed invention and cast the mind back to the time the invention was made (often as here many years), to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art." *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984). See also, M.P.E.P. §2141.01.

2. *A prima facie case of obviousness has not been established*

Proper application of the legal standard of obviousness determination leads to the conclusion that the claimed invention is not obvious over the cited combination of references.

a. Appellant does not contest the Examiner's reading of Bottger et al and Lee et al but submit that the Examiner has mischaracterized the teaching of McCann et al

Appellant readily concedes that, as detailed in the present application including the Background of the Invention section, p53 was a known tumor suppressor protein at the priority date of the present application. Similarly, the interaction of p53 with mdm2, the peptide motif of p53 necessary for mdm2 binding, and potent peptide inhibitors of p53-mdm2 binding were also known. Thus, both WO 93/20238 (e.g. Figures 6 and 7) and WO 96/02642 (*e.g.*, Figures 3, 5, and 6), to which the present application refers extensively, for example at page 1, line 35 – page 2, line 21 of the specification, disclose numerous peptides that are modeled on the mutual binding sites of p53 and mdm2. Bottger *et al.*'s teaching of the consensus sequence PXFXDYWXXL, information about the p53-mdm2 binding sites, and inhibition of the mdm2-p53 interaction as a means to restore p53 function in human tumors, does not add anything significant to the art already acknowledged in the Background of the Invention section of the present application.

The rejected claims do not claim inhibitors of the p53-mdm2 interaction *per se*, or even the fact that certain peptides, such as peptides less than 25 amino acids in length and comprising SEQ ID NO: 3, have the ability to inhibit the interaction between p53 and mdm2. The invention claimed in the present application is based on finding a new class of situations in which

disruption of the p53-mdm2 binding is beneficial. In particular, the invention claimed is based on the unexpected finding that targeting of the mdm2/p53 interaction is expected to be beneficial in cancer cells, in which mdm2 is not overexpressed.

The Examiner has acknowledged that Bottger *et al* does not teach SEQ ID NO: 3. The Examiner has also acknowledged that Bottger *et al* does not teach an *in vitro* method for disrupting the binding of p53 and mdm2 in a population of cancer cells in which mdm2 is not overexpressed, comprising administering a peptide less than 25 amino acids in length comprising SEQ ID NO: 3. However, according to the Examiner, McCann *et al* teach that p53 is suppressed in cancer cells such as breast cancer cells, a majority of which do not overexpress mdm2, which p53 suppression is correlated with the presence of mdm2. Stating it differently, the Examiner says that McCann *et al* teach that in cancer cells which do not overexpress mdm2, such as breast cancer cells, the protein expression of mdm2 is significantly associated with low levels of p53. Based on this reading of McCann *et al* the Examiner asserts that it would have been *prima facie* obvious to use a peptide comprising the 12 amino acid peptide taught by Bottger *et al* to target cancer cells that express p53 and mdm2 to increase p53 function, including those populations of cancer cells that do not overexpress mdm2.

Appellant submits that the Examiner has misunderstood and mischaracterized the teaching of McCann *et al* In particular, the Examiner is wrong to say that McCann *et al* teach that in cancers which do not overexpress mdm2 the protein expression of mdm2 is significantly associated with low levels of p53. To the contrary, the teaching of McCann *et al* is that in those samples which show overexpression of mdm2, p53 is reduced.

As defined in the present application, "cells that do not overexpress mdm2" include all cells in which mdm2 is present at low or normal levels, which can be assessed, *e.g.*, by immunological measurement of mdm2 concentration (page 6, lines 25-32).

A protein can be overexpressed in a cell for a number of reasons. One possible reason is that the gene expressing the protein is amplified. However, overexpression can also take place in the absence of gene amplification, *e.g.*, due to an alteration in the normal regulation of the rate of synthesis of the protein and/or in the rate of destruction of the protein. Gene amplification is only one of a number of different mechanisms by which overexpression of a protein may occur.

McCann *et al* discloses studies into the frequency of Mdm2 overexpression in breast cancers. They look at both gene amplification and protein expression and find that only 7% of these cancers show overexpressed Mdm2.

Indeed, McCann *et al* expressly states that:

> *"Interestingly, at the mRNA level, two studies found increased MDM2 expression with no apparent alteration in MDM2 copy number (Buesco-Ramos et al, 1993; Sheikh et al, 1993), suggesting that mechanisms other than gene amplification may play a role in deregulating the expression of MDM2"* (page 981, right column, first paragraph).

In McCann *et al,* overexpression at the protein level was assessed by immunological measurement. 7% of the samples were found to show 10-50% mdm2 nuclear staining, and these samples were designated as MDM2+ cells. This is in spite the fact that only that 4% of tumor samples assayed have altered mdm2 copy number – as noted above, overexpression of a protein can occur even without gene amplification.

McCann *et al* report that MDM2+ status was significantly associated with low levels of p53 (page 983, left column, last paragraph). As explained above, MDM2+ status indicates overexpression of mdm2. Thus, as noted above, contrary to the Examiner's reading, McCann *et al* teach that p53 levels are reduced in those samples which show overexpression of mdm2.

b. The Examiner failed to properly determine the scope and content of the prior art and the differences between the claimed invention and the prior art when making the rejection

Proper obviousness inquiry requires analysis of the Graham factors, which include determination of: (1) the scope and content of the prior art, (2) any differences between the claimed subject matter and the prior art, and (3) the level of skill in the art.

Appellant submits that McCann has not only been misinterpreted by the Examiner, but has not been read in the proper context of general knowledge in the art at and around the priority date of the present application.

(1) *Level of skill in the art*

The invention is from the field of molecular biology. In this field, the level of ordinary skill has been determined to be high, usually represented by a scientist with a PhD in the relevant field.

Along with a response dated July 17, 2007, Appellant submitted a Declaration of Professor Karen Vousden, Director of the Cancer Research UK (CRUK) Beatson Institute (the "first Vousden Declaration"). A further Declaration by Professor Vousden was submitted with the response dated March 11, 2008 (the "second Vousden Declaration"). Professor Vousden has pursued a distinguished career in cancer research, including study of the function of tumor suppressor protein p53, and her work has led to the recognition of some key features of p53 action that highlight the importance of apoptosis in the tumor suppressor function of p53. Thus, Professor Vousden is unquestionably one of skill in the art pertinent to the invention claimed in the present application.

(2) *Scope and content of the prior art*

Contrary to the Examiner's assertions, the state of the art in the relevant time frame, when taken as a whole, indicated that inhibition of the mdm2/p53 interaction in tumors in which mdm2 is expressed at normal levels (*i.e.*, is not overexpressed) could be non-specifically toxic and consequently would not be a good approach for tumors without Mdm2 overexpression.

The first Vousden Declaration

The present application claims priority from UK application GB9708092.3 filed on April 22, 1997. In Paragraphs 6 through 9 of the first Vousden Declaration, Professor Vousden explains that around that time (and around the 1996 publication date of the McCann paper), it was understood that inhibition of p53 function is important for the development of many cancers. It was also understood that this might be the consequence of a number of different events, including, but not limited to,

1. mutation within the p53 gene;
2. overexpression of Mdm2 – a known negative regulator of p53; and
3. expression of the human papilloma virus E6 protein,

and that these alterations are mostly mutually exclusive. In particular, in Paragraph 7 of the first Vousden Declaration Professor Vousden cites Crook et al, Oncogene 6:873-875 (1991); Scheffner *et al.*, PNAS 88:5523-5527, 1991; Crook *et al.*, Lancet 339:1070-1073, 1992; Leach *et al.*, Cancer Res 53:2231-2234, 1993; and Oliner *et al.*, Nature 358:80-83, 1992 (all of record) as representative of the general knowledge in the art at the relevant time frame that tumors with E6 or Mdm2 overexpression do not have mutated p53 and *via versa*, and that it is only necessary to inactivate p53 through one mechanism.

Professor Vousden cites US 09/403,440 to show that it was also known at the priority date of the present application that: (i) p53 binds Mdm2; (ii) Mdm2 inhibits p53 activity; (iii) inhibition of Mdm2 in normal cells will activate p53; and (iv) Mdm2 is overexpressed in some tumors and this is often associated with retention of wild-type p53. (First Vousden Declaration, Paragraph 8).

Papers published in 1995 (Jones *et al.*, Nature 378:206-208, 1995; Montes de Oca Luna *et al.*, Nature 378:203-206, 1995 – of record) disclosed experimental results showing that deletion of Mdm2 in mice causes embryonic lethality owing to the activation of p53. These findings indicated to those of ordinary skill in the art that while inhibition of Mdm2 can cause activation of p53 in cells where Mdm2 is not overexpressed, this activation is very deleterious to normal tissue. As stated in Paragraph 9 of her first Declaration, these findings suggested to Professor Vousden

> *"that a therapy to inhibit mdm2/p53 would not be selective for tumours where Mdm2 is expressed at normal levels. Instead, the findings suggested that such a therapy could well be non-specifically toxic and consequently would not be a good approach for tumours without Mdm2 over-expression."*

(3) *Differences between the claimed subject matter and the prior art*

Proper obviousness analysis requires that the Examiner occupy the mind of one skilled in the art, who has no knowledge of the claimed invention, is aware of and understands the totality of pertinent knowledge at the time the claimed invention was made, and who is normally guided by the then-accepted wisdom in the art. Appellant submits that an analysis properly conducted respecting these principles must necessarily lead to the conclusion that inhibition of the

mdm2/p53 interaction in cancers that do not overexpress mdm2 would not be an effective approach for activating the tumor suppressor function of p53.

In view of the fact that, as discussed above, at the priority date of the present application there were several mechanisms suggested for the inhibition of p53 function, it was assumed that in tumors that do not overexpress Mdm2, p53 is inhibited by a different mechanism. See, first Voussen Declaration, Paragraph 11.

The results of McCann et al show that only 7% of the tested breast cancers overexpress Mdm2. Since, as discussed above, at the time of the McCann *et al.* paper was published in was generally held that inhibition of the p53/Mdm2 interaction would only be effective in cancers that overexpress Mdm2, the real teaching of the McCann *et al.* paper is that inhibition of the p53/Mdm2 would be an effective treatment approach only in the case of a small proportion of breast cancers, i.e. breast cancers that overexpress Mdm2. As stated in Paragraph 15 of the first Vousden Declaration:

> The Examiner is incorrect in her view that McCann *et al.* teach that *in cancers which do not express mdm2, such as breast cancer cells, the protein expression of mdm2 is significantly associated with low levels of p53.* The study by McCann *et al.* shows that although most breast cancers do not over-express Mdm2, a few of them show elevated Mdm2 expression, and these tumours are significantly associated with low (*i.e.*, wild type) p53 levels. McCann *et al.* state that "*at the protein level, MDM2+ tumours were significantly associated with tumours having low levels of p53 staining.*" (Summary, lines 7/8) This means that those few breast cancers that over-express Mdm2 tend to show low levels of p53 – indicating a retention of wild type p53.

The second Vousden Declaration

In addressing the Examiner's finding that her first Declaration was not persuasive, in her second Declaration Professor Vousden provides a more detailed explanation of the teaching of McCann *et al.*

As explained in Paragraph 4:

> *McCann et al. show that 7/97 tumours have high levels of MDM2 expression (type 2, 10-50% staining) and that these are associated with low levels of p53. Table II of McCann et al. only shows results for tumours that are either amplified for the MDM2 gene (samples 10, 16 and 19 – of which only 2 show type 2 MDM2 staining) or tumours that show high MDM2 protein expression (i.e., 10-50%*

staining) without amplification of the gene (tumours 15, 30, 45, 47 and 60). The authors define these 7 as the MDM2+ tumours and state that these show a significant association with low levels of p53. So tumours with high MDM2 (as defined by type 2 staining) are likely to have low p53 – this is what the authors conclude from their study.

Professor Vousden goes on explaining that, since on page 983 the authors define MDM2+ as "[10-50% of tumor nuclei positive (MDM2+) Table 1]" and "MDM2+" (type 2 staining)," it is absolutely clear that by "MDM2+ tumors" the authors intended to refer to those 7 tumors with high MDM2 expression. Second Vousden Declaration, Paragraph 7. Thus, the Examiner incorrectly interpreted the authors' statement that "MDM2 tumors were significantly associated with tumors having low levels of p53 staining" to mean that "not only those few cancers that over-express Mdm2 tend to show low levels of p53, but those that do not over-express mdm2 also show low levels of p53." (Office Action mailed on September 11, 2007, page 5).

While Professor Vousden recognizes that based on the data shown in Table III of the McCann *et al.* paper, it may be possible to conclude that even the tumours that express lower amounts of MDM2 (type 1 tumours, less than 100% staining) are associated with p53, she notes that the "*authors of McCann et al do not pay much regard to this as they limit their comments to the 7 MDM2+ tumours and one might be reluctant to draw conclusions from data that the authors have chosen not to highlight themselves.*" Second Vousden Declaration, Paragraph 9.

Furthermore, even if one came to such conclusion, the data would still not tell that tumors that do not overexpress MDM2 are also associated with p53, as the Examiner has concluded. As Professor Vousden explains in Paragraph 10 of her second Declaration:

The problem here is that we don't know what normal expression is – and it is quite possible that the type 1 expression is [sic] also represents over-expression of MDM2 compared to normal (there is no normal tissue in the McCann et al. study for comparison). The fact that most of the tumours are apparently negative for MDM2 staining (74/95) does not mean that they don't express any MDM2 – only that it is below the level of detection in this assay. For this reason it is quite hard to interpret the meaning of the less than 10% expression, which is probably why the authors have chosen to base their conclusion on the tumours that clearly over-express MDM2 – that is the type 2 staining ones.

In conclusion, there is nothing in McCann *et al.* that would suggest that disruption of the p53/Mdm2 interaction would be effective in cancers in which Mdm2 is not overexpressed. On the contrary, it is only hindsight, based on the knowledge of the present invention, could have led the Examiner to a different reading of McCann *et al.* and ultimately to the conclusion that the combination of Bottger *et al.* with McCann *et al.* and Lee *et al.* makes obvious the claimed invention.

c. *The combined teaching of Bottger et al., McCann et al. and Lee et al. is that targeting of the mdm2/p53 interaction is of use specifically in cells where mdm2 is overexpressed.*

Bottger *et al.* teaches that the interaction between mdm2 and p53 may be a useful target in cells where mdm2 is overexpressed:

> "In several different tumour systems, including human sarcomas, the mdm2 protein (or its human homolog hdm2) is overexpressed but the p53 gene remains wild type (Oliner *et al,* 1992). This suggests that in these tumours the normal tumour suppressor function of p53 is being inactivated by the presence of abnormally high levels of mdm2. In theory, such tumours should be suysceptible to therapeutic moieties that disrupt the mdm2/p53 interaction, restoring wild type p53 function." (Page 2141, right column, first paragraph, emphasis added).

Accordingly, one of ordinary skill in the art reading Bottger *et al.* would believe that therapeutic targeting of the mdm2/p53 interaction is of use specifically in cells where mdm2 is overexpressed.

As discussed above, there is nothing in McCann *et al.*, when read and understood correctly, to contradict the teaching of Bottger *et al.* or to suggest that targeting of the mdm2/p53 interaction may also be of use when mdm2 is not overexpressed.

This is clearly supported by the conclusion of the first Vousden Declaration that based on the combined teaching of the cited references one of skilled in the art would conclude that *"a therapy based on the 12 amino acid peptide would only be expected to be effective in 7% of breast cancers (i.e., those with over-expressed Mdm2) and would suggest that most breast cancers would not benefit from such therapy."* First Vousden Declaration, Paragraph 16.

Lee *et al.* has been relied on for allegedly teaching that p53 could induce apoptosis and cell cycle arrest, and that loss of -53 function causes increased resistance to chemotherapeutic

agents. This teaching does modify the combined teaching of Bottger *et al.* and McCann *et al.* that targeting of the mdm2/p53 interaction is of use specifically in cells where mdm2 is overexpressed.

CONCLUSION

The rejection of Claims 1, 2 and 8 under 35 U.S.C. §103 as allegedly obvious Bottger *et al.*, 1996 (Oncogene, 13:2141-2147), in view of McCann AH *et al.*, (British J Cancer, 71(5):981-5), and further in view of Lee JM *et al.*, 1995 (Cancer and Metastasis Review, 14(2):149-161) is improper and should be overturned.

The Commissioner is authorized to charge any fees which may be required, including extension fees, or credit any overpayment to Deposit Account No. **50-4634** (referencing Attorney's Docket No. **39749-0001 APC (124263-185762)**.

Respectfully submitted,

Date: March 10, 2009

By: _____
Ginger R. Dreger (Reg. No. 33,055)

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VIII. CLAIMS APPENDIX

Claims on Appeal

1. An *in vitro* method for disrupting the binding of p53 and p53-binding protein mdm2 in a population of cancer cells in which said p53-binding protein mdm2 is not , and comprising overexpressed, comprising administering to the cells a peptide, less than 25 amino acids in length, and comprising SEQ ID NO: 3.

2. The method of claim 1 wherein the p53 is activated for DNA specific binding and transcription.

3. The method of claim 1 wherein the peptide has the property of competing with said p53-binding protein mdm2 for binding p53, but does not inhibit DNA specific binding property of p53.

IX. EVIDENCE APPENDIX

1. Declaration of Professor Karen Vousden under 37 C.F.R. §1.132 dated June 27, 2007.
2. Declaration of Professor Karen Vousden under 37 C.F.R. §1.132 dated March 6, 2008.
3. Crook *et al.*, Oncogene 6:873-875 (1991).
4. Scheffner *et al.*, PNAS 88:5523-5527, 1991.
5. Crook *et al.*, Lancet 339:1070-1073, 1992.
6. Leach *et al.*, Cancer Res 53:2231-2234, 1993.
7. Oliner *et al.*, Nature 358:80-83, 1992
8. Jones *et al.*, Nature 378:206-208, 1995.
9. Montes de Oca Luna *et al.*, Nature 378:203-206, 1995.

Items 1 and 3-9 were submitted with Appellant's Response dated July 17, 2007, and their consideration was indicated in the Office Action mailed on September 11, 2007.

Item 2 was submitted with Appellant's Response dated March 11, 2008, and its consideration was indicated in the Office Action mailed on May 22, 2008.

X. RELATED PROCEEDINGS APPENDIX

None

LIBC/3547601.1

ITEM 1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of:

Serial No: 09/403,440

Filed: 19 January 2000

For: LANE, David

Examiner: Davis, Minh-Tam

Art Unit: 1642

Docket No: 39749-0001APC

SECOND DECLARATION OF KAREN VOUSDEN UNDER 37 CFR' 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, DC 20231

SIR·

I Professor Karen Vousden, hereby declare and say as follows:

1. I am the same Karen Vousden who signed the Declaration dated June 27, 2007, and filed with Applicant's submissions of July 17, 2007.

2. I am familiar with and understand the Office Action dated September 11th, 2007, and this Declaration is made in response to the Examiner's comments made in that Office Action.

3. The Examiner states that the response, including my previous Declaration, was not found persuasive. I have considered the reasons given by the Examiner in support of this conclusion and disagree with her analysis of the McCann et al paper on several points.

4. McCann et al show that 7/97 tumours have high levels of MDM2 expression (type 2, 10-50% staining) and that these are associated with low levels of p53. Table II of McCann et al only shows results for tumours that are either amplified for the *MDM2* gene (samples 10, 16 and 19 – of which only 2 show type 2 MDM2 staining) or tumours that show high MDM2 protein expression (i.e. 10-50% staining) without amplification of the gene (tumours 15, 30, 45, 47 and 60). The authors define these 7 as the MDM2+ tumours and state that these show a significant

association with low levels of p53. So tumours with high MDM2 (as defined by type 2 staining) are likely to have low p53 – this is what the authors conclude from their study.

5. The Examiner makes the statement on page 7 that *'the language "at the protein level, MDM2 tumors were significantly associated with tumors having low levels of p53 staining" (Summary, lines 7-8) indicates that not only those few breast cancers that over-express Mdm2 tend to show low levels of p53, but those that do not over-express mdm2 also show low levels of p53, supra'.*

6. Firstly, it should be pointed out that this is not an accurate quotation from the paper. In fact the paper states *"at the protein level, MDM2+ tumors were significantly associated....."* (emphasis added)

7. On page 983 the authors define MDM2+ as "[10-50% of tumor nuclei positive (MDM2+) Table 1]" and "MDM2+ (type 2 staining)". This makes it absolutely clear that the authors intend the expression "MDM2+ tumors", to mean those 7 tumours with high MDM2 expression. The examiner is mistaken in interpreting this statement as indicting that "not only those few cancers that over-express Mdm2 tend to show low levels of p53, but those that do not over-express mdm2 also show low levels of p53". The authors restrict their comments to only the MDM2+ (i.e. 10-50% staining) tumours.

8. Thus, McCann et al teach that tumours with high MDM2 expression are associated with low p53 and this is only 7% of the cancers.

9. After careful consideration of the results presented in the McCann I believe it may be possible to conclude that even the tumours that express lower amounts of MDM2 (the type 1 tumours, less than 10% staining) are associated with low p53 (there are 14 of these tumours, 12 show low p53 staining and 2 show high). See, in particular Table III. The authors of McCann et al do not pay much regard to this as they limit their comments to the 7 MDM2+ tumours and one might be reluctant to draw conclusions from data that the authors have chosen not to highlight themselves.

10. If we chose to make this conclusion, then it would be true to say that MDM2 expression (either type 1 or type 2) seems to be correlated with low p53. However, this does not tell us that tumours that do not overexpress MDM2 are also associated with low p53 (as the examiner has concluded). The problem here is that we don't know what normal expression is – and it is quite possible that the type 1 expression is also represents over-expression of MDM2 compared to

normal (there is no normal tissue in the McCann et al study for comparison). The fact that most of the tumours are apparently negative for MDM2 staining (74/95) does not mean that they don't express any MDM2 – only that it is below the level of detection in this assay. For this reason it is quite hard to interpret the meaning of the less than 10% expression, which is probably why the authors have chosen to base their conclusion on the tumours that clearly over-express MDM2 – that is the type 2 staining ones.

11. On page 7 of the office action, the Examiner states *"Thus, in view that similar to those few breast cancers having over-expressed mdm2, the presence of mdm2 in most breast cancers which have no over-expression of Mdm2 is also associated with low levels of p53"* and attributes this teaching to that of McCann et al. This idea is repeated several times by the Examiner (e.g. page 8 point 2) and seems to me to form the basis of the rejection of the claims.

12. If I have understood the Examiner's arguments correctly, then I think the Examiner is basing her arguments on the fact that she believes even tumours with no over-expression of MDM2 (i.e. tumours with normal MDM2 expression, which would include most tumours) are correlated with low p53. This is not right and is a misinterpretation of the teaching of McCann et al.

13. As I said above, one does not know what overexpression of MDM2 really is, since we have no normal tissue to compare. It is most likely that both type 2 and type 1 tumours are actually over-expressing MDM2, and the tumours with negative expression are those without over-expression. These MDM2 staining-negative tumours still represent the large majority (74/95 or 78%). So, even if we take the type 1 tumours into account (which again I emphasise the authors did not, suggesting that they are not so sure about the interpretation here), the results still support our previous contention that MDM2 over-expression is not common in breast cancers. Accordingly, the McCann paper teaches that most breast cancers arise without evidence for amplification or overexpression of MDM2 and these cancers are not associated with low levels of p53 (34/74 of them have type 2 and 3 p53 staining).

14. From my reading of McCann et al, I assume that in the 40 tumours without MDM2 staining and with low levels of p53 there must be another mechanism to inactivate p53 – and that inhibiting the p53/MDM2 interaction would not necessarily work in these cases.

15. Taking McCann et al together with the study from Bottger, and without the knowledge of the invention claimed in the present application, I would conclude that a therapy based on the 12 amino acid peptide would only be expected to be effective in 7% (or at the most 22%) of breast

cancers (i.e. those with over-expressed MDM2) and would suggest that most breast cancers would not benefit from such therapy.

16 Despite the comments made by the Examiner in response to my previous Declaration and for the reasons provide above, I maintain by belief that at the time of the McCann et al paper it would have been reasonable to assume that in cells where low or normal levels of MDM2 exist, inactivation of the p53 pathway to allow aberrant tumour growth would have arisen from another mechanism.

17. For the reasons set forth in paragraphs 4 through 17 of this Declaration, I believe the results disclosed in US09/403,440 where they have shown that inhibition of MDM2:p53 has a growth reducing effect in tumour cells in which MDM2 is not over-expressed and consequently is a useful therapy for these cells was surprising given the understanding of the mechanisms involved in p53 function at the time US09/403,440 was filed.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Signed _____ on 6th MARCH 2008

ITEM 2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of:

Serial No: 09/403,440

Filed: 19 January 2000

For: LANE, David

Examiner: Davis, Minh-Tam

Art Unit: 1642

Docket No: 39749-0001APC

DECLARATION UNDER 37 CFR' 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, DC 20231

SIR:

I Professor Karen Vousden, hereby declare and say as follows:

1. I received my Ph.D. in Genetics from the University of London. I carried out postdoctoral fellowships with Professor Chris Marshall at the Institute of Cancer Research in London and Dr. Douglas Lowy at NCI before becoming Head of the Human Papillomavirus Group at the Ludwig Institute for Cancer Research in London in 1987.

2. After joining the ABL-Basic Research Program as Head of the Molecular Carcinogenesis Section in 1995, I was appointed Director of the Molecular Virology and Carcinogenesis Laboratory in 1997 and Interim Director of the ABL-Basic Research Program in 1998. I was then appointed Chief of the Regulation of Cell Growth Laboratory (RCGL), Division of Basic Sciences, NCI.

3. I am currently the Director of the Cancer Research UK (CRUK) Beatson Institute, Garscube Estate, Switchback Road, Bearsden, Glasgow, UK.

4. I have pursued a distinguished career on both sides of the Atlantic untangling the molecular mechanisms that underlie cancer. I have focused on a number of proteins, in particular p53, which act as cancer tumour suppressors in normal cells, but whose functions are disrupted in most human cancers. My work has led to the recognition of some key features of p53 that highlight the importance of apoptosis, or cell death, in the tumour suppressor function of p53. My studies have contributed to the realization that tumour cells have a deregulated pathway for RB (a protein critical for cell cycle regulation) and are more sensitive to p53 driven cell-death than their normal counterparts. Of particular importance is the pivotal work in identifying the role of the Mdm2 protein in regulating p53 activity, which has opened a path to possible re-activation of p53 in some tumour cells, which could provide

a beneficial and therapeutic effect in the treatment of cancer. Recent and selected publications I have authored or co-authored include:

Carter S, Bischof O, Dejean, Vousden KH. (2007). C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. *Nature Cell Biology* 9, 428-435.

Uldrijan S, Pannekoek WJ, Vousden KH. (2007). An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. *EMBO Journal* 26, 102-112.

Wilson JM, Henderson G, Black F, Sutherland A, Ludwig RL, Vousden KH, Robins DJ. (2007). Synthesis of 5-deazaflavin derivatives and their activation of p53 in cells. *Bioorganic & Medicinal Chemistry* 15, 77-86.

Bensaad K, Tsuruta A, Selak MA, Vidal MNC, Nakano K, Bartrons R, Gottlieb E, Vousden KH. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 126, 107-120.

Vousden KH. (2006). Outcomes of p53 activation – spoilt for choice. *Journal of Cell Science* 119, 5015-20.

Bensaad K, Vousden KH. (2005). Saviour and Slayer: the two faces of p53. *Nature Medicine* 11, 1278-1279.

Fogal V, Kartasheva N, Trigiante G, Llanos S, Yap D, Vousden KH, Lu X. (2005). ASPP1 and ASPP2 are new transcriptional targets of E2F. *Cell Death Diff.* 12, 369-376.

Rossi M, De Laurenzi V, Munarriz E, Green DR, Liu YC, Vousden KH, Cesareni G, Melino G. (2005). The ubiquitin-protein ligase itch regulates p73 stability. *EMBO J.* 24, 836-848.

Vousden KH. (2005). Apoptosis. p53 and PUMA: a deadly duo. *Science* 309, 1685-1686.

Vousden KH, Prives C. (2005). p53 and prognosis: new insights and further complexity. *Cell* 120, 7-10.

Weber HO, Ludwig, RL, Morrison D, Kotlyarov A, Gaestel M, Vousden KH. (2005). HDM2 phosphorylation by MAPKAP kinase 2. *Oncogene* 24, 1965-1972.

Yang Y, Ludwig RL, Jensen JP, Pierre S, Medaglia MV, Davydov I, Safiran YJ, Oberi P, Kenten J, Phillips AC, Weissman AM, Vousden KH. (2005). Small molecule inhibitors of HDM3 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell* 7, 547-559.

Yee KS, Vousden KH. (2005). Complicating the complexity of p53. *Carcinogenesis* 26, 1317-1322.

5. I have read and understand McCann et al, British Journal of Cancer (1995) 71, 981-985; Bottger et al 1996, 1996 (Oncogene, 13: 2141-2147) and the comments made by the examiner in the Advisory Action dated 13 December 2006.

6. At the time of publication of the McCann paper, it was understood that inhibition of p53 function is important for the development of many cancers. It was also understood that this might be the consequence of a number of different events such as (but not limited to)

1. Mutation within the p53 gene.
2. Over-expression of Mdm2 – a known negative regulator of p53.
3. Expression of the human papillomavirus E6 protein

7. There was evidence that these alterations are mostly mutually exclusive. In other words, tumours with E6 or Mdm2 over-expression do not have mutated p53 and *vice versa*. The understanding was that it is only necessary to inactivate p53 through one mechanism. (Crook et al, Oncogene 6:873-875, 1991; Scheffner et al, PNAS 88: 5523-5527, 1991; Crook et al, Lancet 339: 1070-1073, 1992; Leach et al, Cancer Res 53: 2231-2234, 1993; Oliner et al., Nature 358: 80-83, 1992).

8. At the time of filing US09/403,440 I was aware that (i) p53 binds Mdm2; (ii) Mdm2 inhibits p53 activity; (iii) inhibition of Mdm2 in normal cells will activate p53; and (iv) Mdm2 is over-expressed in some tumours and this if often associated with retention of wild type p53.

9. Two papers were published in 1995 (Jones et al, Nature 378:206-208, 1995; and Montes de Oca Luna et al, Nature 378:203-206, 1995) that showed that deletion of Mdm2 in mice causes embryonic lethality owing to the activation of p53. Accordingly, I was aware that inhibition of Mdm2 can cause activation of p53 in cells where Mdm2 levels are normal (i.e. not over-expressed) but that this was very deleterious to normal tissue. These findings suggested to me that a therapy to inhibit mdm2/p53 would not be selective for tumours where Mdm2 is expressed at normal levels. Instead, the findings suggested that such a therapy could well be non-specifically toxic and consequently would not be a good approach for tumours without Mdm2 over-expression.

10. Further, at the time of filing US09/403,440, I was aware that some tumours show over-expression of Mdm2. It was expected that these tumour cells would be more sensitive to Mdm2 inhibition than normal cells, so the inhibition of Mdm2 could be useful as a cancer therapy specifically in these cases.

11. It was assumed at that time that in tumours with no over-expression of Mdm2, p53 was inhibited through other, unknown mechanisms. Consequently, it was not known at the time whether inhibition of Mdm2:p53 interaction in such tumours would be an effective therapy, and indeed, there was evidence that such an approach could be very deleterious to normal tissues.

12. Before publication of the McCann paper, it was not clear how many tumour types would show frequent over-expression of Mdm2. The McCann paper examines this question in breast cancers. They look at both gene amplification and protein expression and find that only 7% of these cancers show over-expressed Mdm2. The conclusion from these studies is that inactivation of p53 as a consequence of Mdm2 over-expression occurs in only few breast cancers. As stated by McCann et al *"We conclude that MDM2 gene amplification occurs at a lower frequency in breast cancer than in non-epithelial tumours."* (Summary, lines 8/9)

13. As stated above, at the time of the McCann paper, it was held that inhibition of the p53/Mdm2 interaction would only be effective in cancers that over-express Mdm2. Therefore the McCann paper would teach that this type of therapy would be effective in only a small proportion of breast cancers. There is nothing in this paper to suggest that disruption of the p53/Mdm2 interaction would be effective in cancers in which Mdm2 is not over-expressed.

14. McCann et al also examine p53 expression. They show that, as expected, over-expression of Mdm2 is associated with low levels of p53. It is important to realise that low p53 levels are indicative of the retention of wild type p53 – so this study supports the suggestion made earlier that over-expression of Mdm2 can inhibit p53 - and therefore remove the requirement for a mutation within p53. However, the study suggests that this correlation is not complete, and in some cancers alterations in both Mdm2 and p53 may have occurred.

15. The examiner is incorrect in her view that McCann et al teach that *in cancers which do not express mdm2, such as breast cancer cells, the protein expression of mdm2 is significantly associated with low levels of p53.* The study by McCann et al shows that although most breast cancers do not over-express Mdm2, a few of them do show elevated Mdm2 expression, and these tumours are significantly associated with low (i.e. wild type) p53 levels. McCann et al state that *"at the protein level, MDM2+ tumours were significantly associated with tumours having low levels of p53 staining".* (Summary, lines 7/8) This means that those few breast cancers that over-express Mdm2 tend to show low levels of p53 – indicating a retention of wild type p53.

16. Taking McCann et al together with the study from Bottger, and without the knowledge of the invention claimed in the present application, I would conclude that a therapy based on the 12 amino acid peptide would only be expected to be effective in 7% of breast cancers (i.e. those with over-expressed Mdm2) and would suggest that most breast cancers would not benefit from such therapy.

17. At the time of the McCann et al paper it would have been reasonable to assume that in cells where low or normal levels of Mdm2 exist, inactivation of the p53 pathway to allow aberrant tumour growth would have arisen from another mechanism.

18. For the reasons set forth in paragraphs 6 through 17 of this Declaration, I believe the results disclosed in US09/403,440 where they have shown that inhibition of Mdm2:p53 has a growth reducing effect in tumour cells in which Mdm2 is not over-expressed and consequently is a useful therapy for these cells was surprising given the understanding of the mechanisms involved in p53 function at the time US09/403,440 was filed.

19. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Signed _____ on 27-06-07

ITEM 3

Oncogene (1991), 6, 873-875

SHORT REPORT

p53 point mutation in HPV negative human cervical carcinoma cell lines

Tim Crook[1], David Wrede[1,2] & Karen H. Vousden[1]

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Clinical and experimental evidence is consistent with a key role for transforming human papilloma viruses (HPVs) in the aetiology of anogenital carcinoma. Cervical carcinoma does, however, occasionally occur in the absence of HPV sequences (Riou *et al.*, 1990). We have used a direct cDNA/PCR sequencing protocol to analyse the sequence of p53 mRNA expressed by HPV positive and negative cervical carcinoma cell lines. Six cell lines which contain HPV sequences express p53 mRNA which has wild-type sequence throughout conserved boxes 2, 3, 4 and 5. The two HPV negative cell lines (C33a and HT3) express mutant p53 mRNA. In each case the mutation occurs in an evolutionarily conserved amino acid. Our data suggest that loss of wild-type p53 function is important in development of cervical carcinoma, and that this might be achieved either by mutation within the p53 gene or the presence of a virally encoded p53 binding protein.

Sequences from human papilloma virus types 16, 18, 31, 33 and 35 are frequently found in anogenital carcinomas (reviewed in Vousden, 1989). These HPV types are also able to immortalise primary genital epithelial cells *in vitro* (Pecoraro *et al.*, 1989; Woodworth *et al.*, 1989), strongly suggesting a key role for HPV in the aetiology of these tumours. Immortalisation of primary genital keratinocytes requires expression of both E6 and E7 proteins of the HPV (Hawley-Nelson *et al.*, 1989), and the same viral open reading frames are frequently retained and expressed in carcinomas and carcinoma-derived cell lines (Schneider-Gadicke & Schwarz, 1986; Smotkin & Wettstein, 1986). Both E6 and E7 have been shown to form complexes with the products of cell-encoded tumour suppressor genes; E6 binding to the p53 protein and E7 forming a complex with the product of the RB-1 gene (Werness *et al.*, 1990; Dyson *et al.*, 1989). This ability to encode proteins which bind to both p53 and RB makes the transforming HPV types very similar to the other DNA tumour viruses SV40 and adenovirus. It seems likely that simultaneous interference with both cell proteins is necessary for full transforming potential of the viruses, and recent studies have shown that mutant p53 expression markedly enhances the transforming potential of E7, suggesting cooperation between these two proteins (Crook *et al.*, 1991). Previous reports have demonstrated that mutation in p53 in epithelial cancers is the most common genetic abnormality yet described in these tumours (Iggo *et al.*, 1990; Nigro *et al.*, 1989), and it is possible that loss of wild-type p53 function, either by mutation or by binding to virus encoded proteins, is required for malignant transformation.

Correspondence: T. Crook

Received 6 November 1990; accepted 28 January 1991

Eight previously described cervical carcinoma cell lines, CaSki, HeLa, Mc180, Ms751, C4II, SiHa, C33a and HT3 were obtained from the American Type Culture Collection (Pater & Pater, 1985; Yee *et al.*, 1985). CaSki and SiHa contain sequences from HPV16 (Yee *et al.*, 1985), and HeLa, Ms751, C4II and Mc180 contain either HPV18 or HPV18-related sequences (Yee *et al.*, 1985). C33a and HT3 contain no sequences of any known HPV type, and are assumed to have arisen by HPV-independent mechanisms. All the cell lines were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum. For analysis of p53 sequence, we used a modified direct assymetric polymerase chain reaction (PCR)/cDNA protocol. The principle of assymetric PCR to generate single-strand DNA sequencing templates has been described previously (Gyllensten & Erlich, 1988; Iggo *et al.*, 1990). Total cellular RNA was isolated from sub-confluent flasks of cells by guanidium/phenol extraction, and poly(A)$^+$ RNA was isolated by two cycles of oligo d(T) cellulose affinity chromatography. First-strand synthesis of cDNA was performed with 250 ng of poly(A)$^+$ RNA using random primers and Moloney Murine Leukaemia Virus (MMuLV) reverse transcriptase (BRL). Following first-strand synthesis cDNA was ethanol precipitated from 2 M NH_4OAc and resuspended in 20 μl of water. A 1.4 kb fragment including all the coding sequences of p53 was then amplified from 10 μl of the first-strand reaction by 35 cycles of PCR using primers 1 and 2: P1, TTTCCACGACGGTGACA. P2, AAAATGGCAGGGGAGGG. Each primer also contained terminal EcoR1 restriction sites, in addition to the p53-specific sequences.

After the first round of PCR, the amplified product was divided into four equal aliquots and reamplified with a single PCR primer to generate single-strand DNA for sequencing. The four primers for second round PCR were in pairs, and have been described previously by Iggo *et al.* (1990). One member of each pair was located 5', and one 3' of the conserved boxes 2 and 3, or conserved boxes 4 and 5 of the p53 cDNA (Soussi *et al.*, 1987; Iggo *et al.*, 1990). In this way, the cDNA sequence of both strands of the four conserved boxes could be determined in a single sequencing experiment. The primer sequences were: **Boxes 2 and 3:** 5' primer: CAGCTCCTACACCGGCGGCCCCTGCACCAG; 3' primer: GAGCCAACCTCAGGCGGCTCATAGGGCACC. **Boxes 4 and 5:** 5' primer: TAGTGTGGTGGTGCCCTATGAGCCG; 3' primer: GTGGGAGGCTGTCAGTGGGGAACAA. Second round PCR was performed for 25 cycles, and the amplified single-strand DNA was extracted with phenol/chloroform and cleaned by three cycles of ultrafiltration in a centricon 100 unit. The cleaned product was finally precipitated from 2 M NH_4OAc by 50% isopropanol, washed with

75% ethanol and resuspended in sequencing buffer (40 mM Tris.Cl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl). Sequencing was performed using sequenase and the corresponding primer from the paired PCR primers described above. Sequencing reactions were resolved on 6% polyacrylamide gels.

Initially, we analysed amplified cDNA from p53 conserved boxes 2 and 3 for the presence of point mutations. In all eight cell lines the sequence of this segment of the p53 cDNA was wild-type. We then analysed conserved boxes 4 and 5. In the HPV containing cell lines (CaSki, SiHa, C4II, HeLa, Me180 and Ms751) the sequence of boxes 4 and 5 was wild-type. In the HPV − ve cell lines C33a and HT3, we consistently identified single point mutations in each p53 cDNA (Figure 1). In the case of C33a, the point mutation (CGT–TGT) occurs at amino acid 273, and converts the wild-type arginine to cysteine. Mutation of this amino acid (to histidine) occurs frequently in colorectal carcinoma cell lines (Rodrigues *et al.*, 1990). In HT3, the point mutation (GGC–GTC) occurs at amino acid 245 and converts the wild-type glycine to valine. In both cases, the point mutation occurs in amino acids which are evolutionarily conserved (Soussi *et al.*, 1987).

We are confident our data represent genuine mutations since (a) we observed the same mutation in cDNA prepared from two completely independent preparations of poly(A)$^+$ RNA, (b) in each experiment we sequenced both strands of the cDNA, (c) direct sequencing of PCR products means that it is highly unlikely that errors due to misincorporation by the Taq polymerase will be represented in the final sequencing gels (Gyllensten & Erlich, 1988). We have demonstrated an interesting correlation between the presence of HPV sequences and the absence of cellular p53 point mutations. Since we have only sequenced conserved boxes 2, 3, 4 and 5 of expressed p53 mRNA, it remains possible that mutations exist outside these regions. However, previous studies in other carcinomas have demonstrated that the large majority of p53 mutations occur within the conserved regions which we have examined in the present study.

Our data are consistent with the hypothesis that interference with the function of wild-type p53 plays a critical role in the aetiology of cervical neoplasia, and the exclusive nature of the presence of HPV or p53 mutations can be interpreted as further evidence for a direct role for HPV in the formation of the majority of cervical tumours. In the presence of transforming HPV types, loss of wild-type p53 function may occur via complex formation with E6, which could lead to enhanced degradation of the complexed wild-type protein, and the lack of p53 point mutations in the HPV + ve cell lines which we now report is consistent with such a role for E6/p53 complex formation. In the absence of HPV-encoded functions, loss of p53 wild-type function appears to occur via point mutation. In either case, the net result is presumed to be loss of wild-type p53 function. It will clearly be important to determine whether the point mutations we describe in

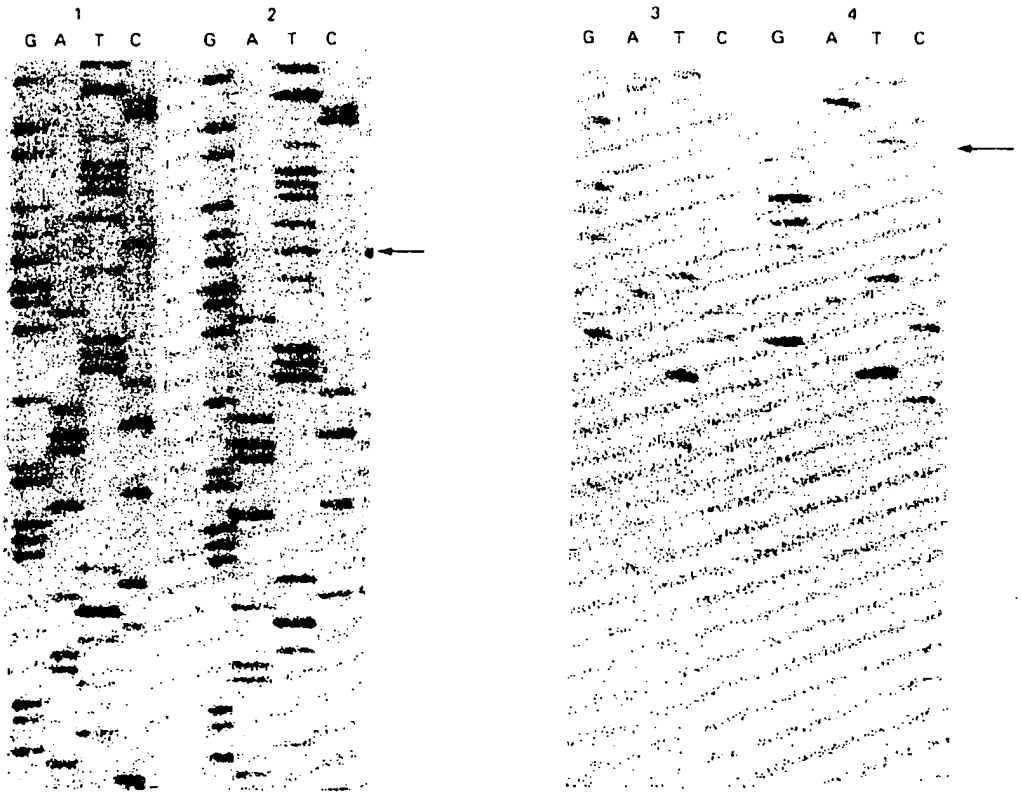

Figure 1 Mutant p53 sequences in HPV − ve cervical carcinoma cell lines. Lane 2: C33a, point mutation, CGT–TGT is arrowed. Compare with Lane 1, Me180, wild-type sequence. Lane 4: HT3, point mutation, GGC–GTC, is arrowed. Lane 3: Ms751, wild-type

the present report do indeed confer a transforming phenotype to p53, and whether these mutant p53 proteins can functionally substitute for E6 expression in appropriate *in vitro* systems. It will also be of interest to determine whether mutations occur in regions of p53 which we have not analysed in the present study. However, it appears highly likely that interference with the p53-related pathway plays a key role in cervical neoplasia.

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ITEM 4

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Medical Sciences

The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines

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ABSTRACT **Human cervical carcinoma cell lines that were either positive or negative for human papillomavirus (HPV) DNA sequences were analyzed for evidence of mutation of the p53 and retinoblastoma genes. Each of five HPV-positive cervical cancer cell lines expressed normal pRB and low levels of wild-type p53 proteins, which are presumed to be altered in function as a consequence of association with HPV E7 and E6 oncoproteins, respectively. In contrast, mutations were identified in the p53 and *RB* genes expressed in the C-33A and HT-3 cervical cancer cell lines, which lack HPV DNA sequences. Mutations in the p53 genes mapped to codon 273 and codon 245 in the C33-A and HT-3 cell lines, respectively, located in the highly conserved regions of p53, where mutations appear in a variety of human cancers. Mutations in *RB* occurred at splice junctions, resulting in in-frame deletions, affecting exons 13 and 20 in the HT-3 and C-33A cell lines, respectively. These mutations resulted in aberrant proteins that were not phosphorylated and were unable to complex with the adenovirus E1A oncoprotein. These results support the hypothesis that the inactivation of the normal functions of the tumor-suppressor proteins pRB and p53 are important steps in human cervical carcinogenesis, either by mutation or from complex formation with the HPV E6 and E7 oncoproteins.**

Cervical cancer is one of the leading causes of female death from cancer worldwide with ≈500,000 deaths per year. Epidemiologic studies have implicated a sexually transmitted agent in the etiology of cervical cancer, and laboratory studies over the past decade have established a strong association between certain human papillomaviruses (HPVs) and cervical cancer and several other anogenital carcinomas (for review, see ref. 1). Over 65 different HPVs have now been described, and ≈20 of these have been associated with anogenital lesions (2). A subgroup of these viruses, including HPV types 16, 18, 31, 33, and 39, have been etiologically implicated in cervical carcinogenesis because they are found in a high percentage of the cancers and because the benign lesions with which these viruses are associated are precursors for malignant progression.

Additional evidence that HPVs have an etiologic role in cervical neoplasia derives from the analysis of the properties of the viral gene products expressed in these cancers. The viral *E6* and *E7* genes are regularly expressed in the HPV-positive tumors and cervical carcinoma cell lines (3–6), and both genes have transforming properties. *E7* alone can transform established rodent cells, such as NIH 3T3 cells (7–12), and can cooperate with an activated *ras* oncogene to transform primary rat cells (8, 13). The transforming potential of *E6* was revealed by studies showing that efficient immortalization of primary human keratinocytes or human fibroblasts required the combination of *E6* with *E7* (14–16).

Insight into the mechanisms by which DNA tumor viruses transform cells has come from the recognition that the virus-encoded oncoproteins interact specifically with important cell regulatory proteins. The E7 protein of the genital tract HPVs, similar to the adenovirus E1A proteins (17) and the large tumor antigens of the polyomaviruses (18, 19), can complex with the product of the retinoblastoma tumor-suppressor gene pRB (20, 21). The E7 proteins of the "high risk" HPVs, such as HPV-16 and HPV-18, bind pRB with ≈10-fold higher affinity than do the E7 proteins of the "low risk" HPV types 6 and 11, and this difference in binding affinity correlates with the transforming potential of the different E7 proteins (21). Like simian virus 40 (SV40) large tumor antigen and adenovirus 5 E1B (22–24), the E6 protein of the "high risk" HPVs can complex with the p53 protein (25), which is now also recognized as having tumor-suppressor properties (26, 27). Because of the tumor-suppressor properties of pRB and p53, the oncogenic effects of these viruses are believed to result, at least in part, from these specific interactions.

In this study we have examined the status of the pRB- and p53-encoding genes in a series of human cervical carcinoma cell lines previously analyzed for HPV DNA. In each of the two HPV-negative cell lines, elevated levels of p53 protein were found. Because mutations in the gene encoding p53 can cause accumulation of ostensibly inactive p53 aggregates, the p53-encoding genes were sequenced and found to be mutated. In contrast, the levels of p53 protein in five HPV-positive cell lines were low, and sequence analysis of the p53 cDNAs revealed no mutations. pRB appeared normal in the HPV-positive cell lines, in that normal-sized phosphorylated as well as hypophosphorylated forms of the protein were detected by immunoblot analysis. In the HPV DNA-negative cell lines, however, mutations in the pRB-encoding gene were found that affected the capacity of the encoded proteins to be phosphorylated and complexed with adenovirus E1A, characteristics of pRB inactivation seen in a variety of other tumors. These results support the hypothesis that the normal functions of pRB and p53 proteins are abrogated in human cervical cancer, either by mutation of the genes themselves or as a consequence of specific interaction of these proteins with the E6 and E7 oncoproteins.

MATERIALS AND METHODS

Cell Lines. The following human cervical carcinoma cell lines were obtained from the American Type Culture Collection: C-33A, HT-3, ME-180, SiHa, C-4II, HeLa, and CaSki (Table 1). The Saos-2 cell line was originally derived from a human osteosarcoma and was obtained from Stephen Friend (Harvard Medical School). Nontransformed primary and secondary human foreskin keratinocytes (HFKs) were prepared and maintained as described (29). The SV40-immortalized HFK cell line (HFK/SV40) was obtained from Richard Schlegel (30). The HFK/1321 cell line was immortalized by the HPV-16 *E6/E7* genes expressed from the

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Abbreviations: SV40, simian virus 40; nt, nucleotide(s); HFK, human foreskin keratinocyte; HPV, human papillomavirus.

Table 1. Human cervical carcinoma cell lines

Cell line	HPV DNA	HPV RNA	Ref.
HeLa	HPV-18	Yes	3
C-4II	HPV-18	Yes	3
SiHa	HPV-16	Yes	4, 28
CaSki	HPV-16	Yes	4, 28
ME-180	HPV*	Yes*	28
C-33A	Negative	No	28
HT-3	Negative	No	28

*The ME-180 cell line was reported as containing HPV DNA detected with a HPV-18 DNA probe (28). Further analysis of this cell line has revealed transcriptionally active HPV sequences that are more closely related to HPV-39 than to HPV-18 but for which the HPV type number is yet unassigned (Elizabeth Schwarz, personal communication).

human β-actin promoter (14), the HFK/1319 cell line was immortalized by a plasmid (p1319) containing the HPV-16 early region expressed from the human β-actin promoter (K.M., unpublished work), and the HFK/698 (29) and HFK/769 cell lines were immortalized by cloned HPV-16 DNA.

Immunologic Procedures. For immunoblotting, cellular protein lysates were prepared from 80% confluent cells in lysis buffer (1% Nonidet P-40/100 mM NaCl/2 mM EDTA/20 mM Tris, pH 8.0) containing phenylmethylsulfonyl fluoride (0.01%)/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)/NaF (5 mM), and sodium orthovanadate (1 mM) at 0°C for 30 min. Lysates were cleared by centrifugation at 15,000 × *g* for 15 min and stored at −80°C. Protein concentrations were determined by the Bio-Rad protein assay. Samples (100 μg) were analyzed by SDS/PAGE followed by immunoblotting (31). The mouse monoclonal antibodies Mh-Rb-02 (PharMingen, San Diego) and PAb1801 (32) (marketed as AB2; Oncogene Sciences, Mineola, NY) were used to detect pRB and p53, respectively. An ^{125}I-labeled sheep anti-mouse antibody (Amersham) was used for detection.

PCR Analysis, Cloning, and DNA Sequencing. Cytoplasmic RNA, prepared by using standard procedures (33), served as a template for cDNA synthesis. Reverse transcription was followed by PCR amplification, according to the suggestions of the manufacturer (Cetus). The primer for reverse transcription of p53 mRNA extended from nucleotide (nt) 1015 to nt 996 with a *Hin*dIII site at the 5′ end for subsequent cloning, where nt 1 is the adenine of the ATG initiation codon. The opposing primer used for PCR amplification extended from nt 296 to nt 315 and contained a *Sal* I site at its 5′ end. The RB sequences for the RB primers used in this study use the nucleotide numbering system of Friend *et al.* (34) and are as follows: exon 12 (sense), nt 1171–1190; exon 13 (sense and antisense), nt 1300–1321; exon 16 (antisense), nt 1470–1494; exon 16/17 (sense), nt 1489–1508; exon 18/19 (sense and antisense), nt 1807–1831; exon 20 (sense and antisense), nt 2005–2026; exon 21 (antisense), nt 2123–2145; and exon 22/23 (antisense), nt 2321–2339. For genomic analysis additional primers derived from RB intron sequences (35) were used: CACAGTATCCTCGACATTGATTTCTG (intron 12, sense), CGAACTGGAAAGATGCTGC (intron 13, antisense), CTCTGGGGGAAAGAAAAGAGTGG (intron 19, sense). All RB primers contained guanine- or cytosine-rich regions at their 5′ ends and either *Sal* I (sense primers) or *Bam*HI (antisense primers) cloning sites. For PCR analysis of genomic DNA, 250 ng to 1 μg of cellular DNA was used as a template under the conditions suggested by the manufacturer (Cetus). PCR products were cloned into pUC19 and pGEM-1 vectors (Promega), and sequence analysis was carried out on several clones from independent PCR reactions by using modified T7 DNA polymerase (Sequenase; United States Biochemical).

RESULTS

The cervical carcinoma cell lines examined in this study and their status with respect to the presence and the expression of HPV DNA are summarized in Table 1. To verify that the C-33A and HT-3 cell lines were indeed HPV negative, DNA from each cell line was further examined by filter hybridization under nonstringent conditions (36) and by PCR with consensus primers. Southern blot hybridization at a melting temperature (t_m) of −45°C with mixed HPV DNA probes, and PCR analysis with consensus HPV primers capable of detecting a wide spectrum of genital-tract HPV types (37) also failed to reveal any HPV DNA in C-33A or HT-3 cell lines (data not shown).

Analysis of pRB in Human Cervical Carcinoma Cell Lines. At least part of the transforming capacity of the E7 protein has been proposed to be a consequence of its ability to interact with pRB protein, thus abrogating the function of pRB as a negative regulator of cell proliferation. This hypothesis leads to the prediction that mutations in *RB* would not, therefore, be of selective advantage in HPV-positive cancer cell lines. Furthermore, if *RB* were an essential target in human cervical cancer, one might expect *RB* inactivation in the HPV-negative cervical carcinoma cell lines to be achieved by other means, such as mutation of the *RB* gene. An immunoblot analysis of pRB was, therefore, done on the human cervical carcinoma cell lines (Fig. 1). In each HPV-positive cervical cell line normal pRB was detected with evidence of both hypophosphorylated and hyperphosphorylated forms of the protein (indicated as pRB and ppRB in Fig. 1). In contrast, a protein with an altered mobility was detected in each of the HPV-negative cell lines C-33A and HT-3. A small amount of normal forms of the RB proteins could also be detected in the HT-3 cells, possibly from admixture of some cells with normal RB proteins (see below). The faster migrating forms of pRB seen in each of these two HPV-negative cell lines appear as single bands, indicating the presence of only the hypophosphorylated form of pRB. Complex formation with adenovirus E1A *in vitro*, an attribute of the wild-type protein, could not be detected, providing further evidence that pRB protein was abnormal in each of these cell lines (data not shown).

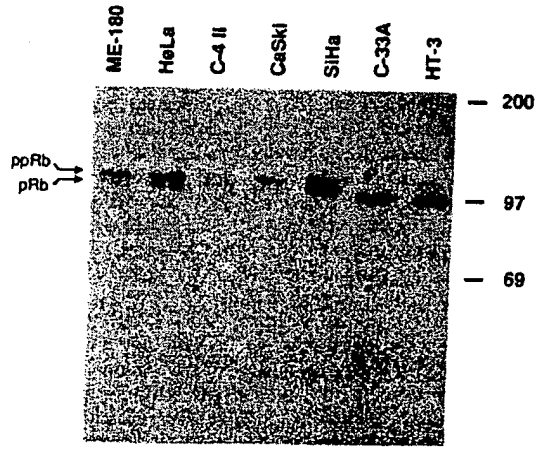

FIG. 1. Immunoblot analysis of pRB in cervical carcinoma cell lines. One hundred micrograms of protein extract of each indicated cell line was separated on SDS/7.5% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The RB protein was detected with the monoclonal mouse antibody Mh-Rb-02. Positions of the pRB protein in hypophosphorylated (indicated here as pRB) or hyperphosphorylated (ppRB) forms.

Structure of RB mRNA in C-33A and HT-3 Cell Lines. To verify that C-33A and HT-3 cell lines express mutant pRB, cDNA representing the pRB mRNA was examined by PCR analysis. Altered forms of pRB protein defective in their ability to be phosphorylated and to complex adenovirus E1A have been demonstrated in a variety of human cancers. Because mutations that affect these properties of pRB have been mapped to genomic sequences encoding exons 13–22 (38–40), PCR primers were designed to examine these exons in the cDNAs. cDNA from HeLa cells, which contain normal pRB, was used as control.

Analysis of PCR products for the C-33A cell line revealed a small deletion, evidenced by the shorter PCR product seen with the E18/19 primer and either the E21 or E20 primer. Sequence analysis of the PCR product revealed a 12-base deletion at the 5′ end of exon 20, resulting in an in-frame deletion of four amino acids (Fig. 2). A similar analysis of the HT-3 RB cDNA revealed that exon 13 was entirely deleted from the cDNA (Fig. 2).

Determination of the Genomic Mutation in *RB* in C-33A and HT-3 Cell Lines. To determine the basis for each of the altered cDNAs in these two cell lines, we examined the genomic sequences surrounding the junctions for the exon 20 splice-acceptor site in C-33A and for the exon 13 splice-donor and -acceptor sites in HT-3; exon skipping is commonly associated with splice-junction mutations (41). A single G → A mutation was found in the exon 20 splice acceptor in C-33A cells, and an A → G mutation was found in the exon 13 splice donor in HT-3 cells (Fig. 3). These mutations were found in multiple independent clones of PCR-amplified segments of genomic DNA from these cell lines, indicating that the mutations did not represent PCR-generated artifacts. One of five clones from the HT-3 cells contained a wild-type RB sequence, supporting the observation that the cell line is probably not clonal and contains some cells expressing normal pRB, seen faintly in Fig. 1. No mutation was found at the exon 13 splice acceptor in HT-3 cells. Thus, in each of the HPV-positive lines, pRB was normal but apparently complexed with E7, whereas in the HPV-negative cell lines examined, pRB was present in a mutant form.

Analysis of p53 in Cervical Carcinoma Cell Lines. Levels of p53 protein were examined in these cell lines by immunoblot analysis with mouse monoclonal antibody 1801 to human p53

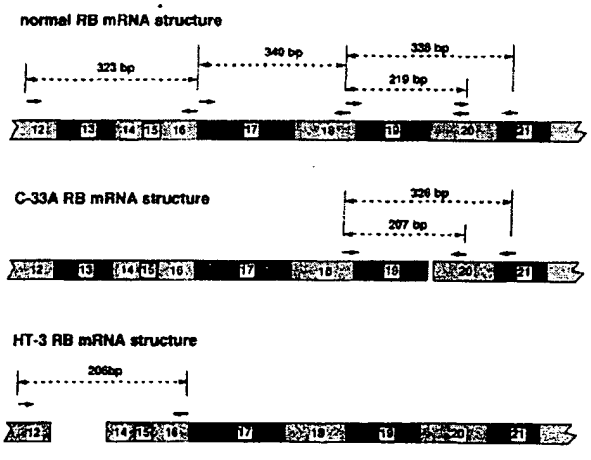

FIG. 2. Schematic representation of mutations in RB mRNA determined from PCR analyses and DNA sequence analysis. The structure of normal RB mRNA is shown at top. The RB mRNA from C33-A cells has a deletion of 12 base pairs (bp) at the beginning of exon 20, resulting in in-frame deletion of four amino acids. The RB mRNA from HT-3 cells contains a precise deletion of exon 13, resulting in in-frame deletion of 39 amino acids.

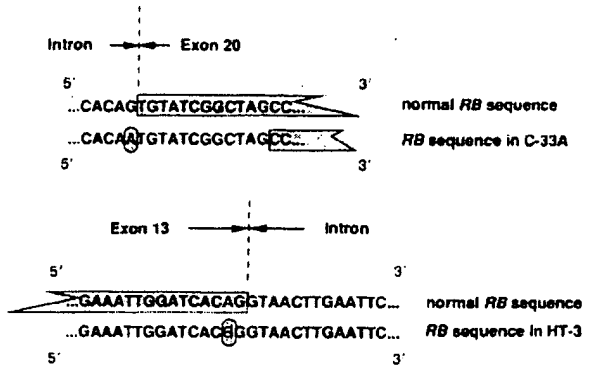

FIG. 3. Genomic mutations in the *RB* gene in C-33A (*Upper*) and HT-3 (*Lower*) cell lines. In the C-33A cell line a G → A mutation occurs at the intron/exon 20 splice junction. A new cryptic splice acceptor is used 12 bases downstream. The HT-3 cell line contains an A → G mutation at the −2 position of the 5′ splice junction, thus skipping exon 13.

(32). A specific band of p53 was detected in HFKs and in each of the cervical carcinoma cell lines (Fig. 4*A*). Saos-2 cells were included in this analysis as a negative control because they do not contain or express p53 (42). p53 levels in the

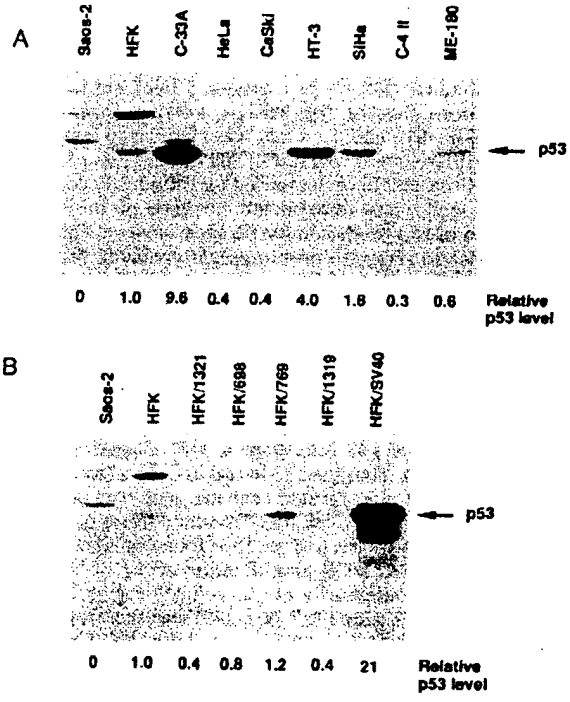

FIG. 4. Immunoblot analysis of p53 in human cervical carcinoma cell lines and in HFKs. Equal amounts of protein (100 μg) extracted from several human cervical carcinoma cell lines (*A*) or HFK cell lines immortalized by cloned viral plasmids (*B*) were separated through SDS/10% polyacrylamide gel before electroblot transfer to nitrocellulose filters. In these experiments, the human osteosarcoma cell line (Saos-2), which does not contain p53 (42), and secondary cultures of HFKs, which express very low levels of p53, served as controls. The p53 protein was detected using mouse monoclonal antibody PAb 1801 (32), as described. An AMBIS scanner (AMBIS Systems, San Diego) was used for measurement, and p53 levels are expressed relative to the level in nonimmortalized HFKs. In subsequent cultures of HT-3 cells, the p53 level was <4.0 in relative level, seen in the experiment of *A* with a relative level of 2.0.

carcinoma cell lines were measured and are expressed relative to the p53 level detected in HFK cells, which is extremely low, as it is in most primary cells. A previous analysis of p53 in HeLa cells reported no detectable protein despite the presence of translatable mRNA (43); however, very low p53 levels could be demonstrated in HeLa cells in this analysis. This result was similar to that seen with the four other HPV-positive cell lines examined (Fig. 4*A*), in which p53 levels detected ranged from 0.3- to 1.8-times that found in secondary cultures of HFKs. Thus, in nontransformed HFKs as well as in HPV-positive cervical carcinoma cell lines, the levels of p53 are very low. The low p53 levels in these cell lines contrasted with those found in the two HPV-negative cervical carcinoma cell lines studied. In the experiment depicted in Fig. 4*A*, p53 levels in C-33A and HT-3 cell lines were 9.6- and 4.0-times that of HFKs.

Analysis of p53 and pRB in SV40 and HPV-Immortalized Keratinocytes. These results with the cervical carcinoma cell lines prompted examination of p53 levels in a series of human keratinocyte lines immortalized by HPV-16 and by SV40. The E6 oncoprotein of the HPV types associated with cervical cancer can exist in a complex with p53 in *in vitro* assays (25) and can promote its degradation *in vitro* (44). In contrast, SV40 large tumor antigen, which also complexes p53, increases the half-life and steady-state levels of p53 in transformed cells (45). The levels of p53 were therefore measured in a series of four independent HFK cell lines immortalized by different plasmids expressing the full HPV-16 genome or portions of the HPV-16 early region containing *E6* and *E7*. The levels of p53 were measured directly from the immunoblot and compared with the level in nontransformed HFKs. The levels observed for the individual HPV-16-immortalized lines did not differ markedly from that of the HFKs, varying from 0.4- to 1.2-times (Fig. 4*B*), and, as such, were similar to levels seen in HPV-positive cancer cell lines. The finding that p53 levels in HPV-positive cell lines decreased only modestly compared with HFK cells was unexpected because of the striking degradation of p53 promoted by E6 seen *in vitro*. This result suggests that *in vivo* p53 proteolysis is probably regulated, and perhaps the effect of E6 on this process is restricted to certain times in the cell cycle. As anticipated, the p53 level in the SV40-immortalized HFKs was markedly elevated over that of the nonimmortalized HFKs (45). Immunoblot analysis of pRB in these immortalized cell lines revealed normal levels of phosphorylated and hypophosphorylated forms of pRB (data not shown).

Determination of p53 Mutations in HPV-Negative Cervical Cancer Cell Lines. That the p53 levels in C-33A and HT-3 cell lines were elevated suggested that the gene was potentially mutated in each of these cell lines: mutated forms of p53 often have extended half-lives and are thus found at higher steady-state levels (46–48). PCR amplification of the cDNA region spanning the evolutionarily conserved p53 region often mutated in cancers (codons 117–309) (49) was therefore carried out, and multiple clones were sequenced. Point mutations resulting in amino acid substitutions were found in the p53 cDNAs in C-33A and HT-3 cell lines within this region, affecting codons 273 and 245, respectively. A CGT → TGT at codon 273 in C-33A cells resulting in an amino acid change of Arg → Cys was found, and a GGC → GTC at codon 245 resulting in a Gly → Val substitution was found in HT-3 cells. Multiple independent clones verified these mutations. These p53 mutations have also been independently noted in each of these two cell lines (T. Crook and K. Vousden, personal communication).

Despite the low levels of p53, the p53 genes might also be mutated in the HPV-positive cell lines. The same conserved region of p53 analyzed above was therefore amplified and sequenced from the cDNA of each of the five HPV-positive cell lines. No p53 gene mutations were found.

DISCUSSION

Approximately 85% of human cervical cancers harbor HPV DNA sequences (1, 50), and the viral E6 and E7 oncoproteins are generally expressed within these tumors (3–6). The tumor-suppressor proteins pRB and p53, which can be complexed by the E7 and E6 oncoproteins, respectively (20, 21, 25), may be relevant targets of the HPVs. Indeed, mutations that inactivate or alter the functions of each of these genes characterize many different human cancers. Mutations in *RB* that eliminate expression of the gene or result in a truncated or functionally altered product have been demonstrated in a variety of human cancers other than retinoblastomas, including sarcomas, small cell carcinomas of the lung, and breast cancers (51). Mutations in the p53 gene have been similarly detected in a high percentage of colon, breast, lung, brain, and esophageal human cancers (49).

The availability of a series of HPV-positive and HPV-negative human cervical carcinoma cell lines provided the opportunity to evaluate whether or not genetic events that altered pRB and p53 might play a role in this cancer. The results were consistent with the hypothesis that pRB and p53 regulatory functions are commonly annulled in human cervical cancers, either by mutation in the HPV-negative cases or as a consequence of their complex formation with the HPV E6 and E7 oncoproteins.

The binding of viral oncoproteins to pRB is thought to functionally inactivate its tumor-suppressor activity. The active form of pRB appears to be the hypophosphorylated form of the protein (52–54), and it is this form that is preferentially found in complex with SV40 large tumor antigen (55) and HPV E7 (K.M., unpublished observation). By this model, one assumes that the functional form of pRB is bound in an inactive complex, no longer inhibiting cellular proliferation.

In human retinoblastomas and other sporadic cancers, mutations in *RB* have been compiled and found to map to regions of the cellular protein involved in complexing with the viral oncoproteins (38, 39). The mutated forms of pRB found in cancer cells can no longer complex with viral oncoproteins, suggesting that the former proteins may also be deficient in their ability to associate with the normal cellular targets of pRB (38, 39, 56). In addition, these mutated forms of pRB are impaired in their ability to be phosphorylated (38, 39, 56). The mutated forms of pRB in C-33A and HT-3 cells have these same characteristics in that they were not phosphorylated and could not complex with adenovirus E1A. The mutations in each of the cell lines mapped to splice junctions affecting exons 13 and 20, respectively, and fall within the domains of pRB necessary for complexing the viral oncoproteins. The splice-acceptor mutation in C-33A cells leads to the in-frame deletion of four amino acids through the use of an alternate acceptor site 12 nt downstream. The splice-donor mutation in HT-3 cells (AGGT → GGGT) results in the precise deletion of exon 13 from the mRNA. Mutations in the 5′ splice junctions that result in exon skipping have been previously described at the −1, +1, and +2 positions, but to our knowledge this is the only example of a naturally occurring mutation with this effect at the −2 position (41).

The complex formation between the viral oncoproteins and p53 is also thought to inactivate the normal function of p53 in regulating cell proliferation. In SV40 and adenovirus 5-transformed cells, association of the virus-encoded oncoproteins and p53 increased half-life and steady-state levels of p53 (45). The association of HPV-16 or HPV-18 E6 in complex with p53 has been demonstrated *in vitro* (25). Because of this association *in vitro*, p53 is targeted for degradation through the ubiquitin-dependent proteolysis system (44). As anticipated, the p53 level in SV40-immortalized keratinocytes was very high, and the levels in the several lines of HPV-immortalized keratinocytes examined were quite low, al-

though still detectable. Low levels of p53 were also found in HPV-positive cervical carcinoma cell lines, indicating that the E6 association with p53 does not cause an increase in its steady level *in vivo*. Relative to nonimmortalized HFKs, the steady-state level of p53 measured in Fig. 4 was lower in four of five HPV-positive cervical carcinoma cell lines examined and in three of four independent HPV-immortalized HFK lines. Assuming that the E6-promoted degradation of p53 seen *in vitro* is of physiological significance, these data indicate that not all cellular p53 is targeted by E6. This discrepancy between the marked *in vitro* degradation of p53 promoted by E6 and the modestly decreased levels of p53 seen *in vivo* is yet to be understood.

The elevated levels of p53 seen in the C-33A and HT-3 cell lines suggested that this gene might be mutated in each of these two HPV-negative cell lines; this possibility was confirmed by direct sequence analysis of cDNA from each line. The mutations affected codons 245 and 273 and, as such, map to an evolutionarily conserved domain in which many mutations have been detected in a variety of human cancers (49).

This study provides evidence that p53 and pRB are relevant targets in cervical carcinogenesis. Inactivation of these two cellular tumor-suppressor proteins through their interaction with E6 and E7 may be the functional equivalent of specific mutations in the p53 and *RB* genes. Some mutations in p53 may actually result in a gain of function (57), something that may not be achieved by the E6/p53 interaction. Furthermore, such activating p53 mutations could even be associated with neoplastic progression in some HPV-positive cancers if the mutated p53 were not able to complex with E6 and were therefore not targeted for degradation.

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ITEM 5

possible protective measure for future pregnancies in severely Rh(D)-alloimmunised women.

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Clonal p53 mutation in primary cervical cancer: association with human-papillomavirus-negative tumours

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Analyses of cancer cell lines and of anal cancers suggest an inverse correlation between infection with human papillomavirus (HPV) and somatic mutation of the p53 tumour-suppressor gene. We have investigated this association in primary cervical tumours.

Tumour-tissue samples from 28 women with primary cancer of the cervix were analysed for presence of HPV sequences and for somatic mutations of the p53 gene. Southern blot analysis and the polymerase chain reaction (PCR) showed that 25 of the tumours contained HPV sequences; 20 were HPV16 positive and 5 HPV18 positive. 17 tumours subjected to restriction fragment length polymorphism analysis for the short arm of chromosome 17 showed no evidence of allelic deletion. Sequencing of the entire coding region of the p53 gene by asymmetric PCR detected heterozygous point mutations in only 3 HPV-negative tumours. By contrast, in 21 HPV-positive cancers the p53 sequence was wild-type throughout.

Our data indicate that loss of wild-type p53 function is important in the pathology of cervical cancer and that in the absence of an HPV-encoded gene product that mediates loss of p53 function, somatic mutation of the gene is required. This pattern of p53 mutation may partly explain the apparently worse prognosis of HPV-negative cervical cancers.

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Introduction

The development of cervical and anal cancers has been linked with infection by human papillomaviruses (HPVs), most commonly HPV16 or 18.[1,2] Experiments that show cell immortalising and transforming activities encoded by HPV16 and 18 support the hypothesis that infection with these viruses contributes to malignancy.[3,4] The virally encoded oncoproteins E6 and E7 both form a complex with cell-encoded protein products of tumour-suppressor genes, E6 binding to the cell p53 protein[5] and E7 to the retinoblastoma gene product (RB).[6] It seems likely that these viral-host protein interactions result in loss of the negative growth control normally exerted by p53 and RB. E6 can direct the rapid proteolytic degradation of p53 and

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this activity is dependent on the ability of the two proteins to form a complex.[7,8] The consequence of E6 expression following HPV infection is likely, therefore, to be a loss of functional wild-type p53 protein within the cell. However, primary cervical tumours do occur in the absence of HPV infection and two studies [9,10] indicate a worse prognosis for HPV-negative cancers.

Our analyses of cervical cancer cell lines and a small series of anal cancers have suggested an inverse correlation between presence of HPV sequences in these cancers and presence of somatic p53 mutation. Six HPV-positive cervical cancer cell lines expressed wild-type p53, whereas two apparently HPV-negative lines expressed p53 mRNA with point mutation in evolutionarily conserved codons.[11] In addition, analysis of HPV-positive primary anal cancers revealed that these retained wild-type p53 coding sequences, whereas rare HPV-negative tumours all contained heterozygous p53 point mutation in conserved codons.[12] These data imply that loss of wild-type p53 function is a critical event in the pathology of anogenital cancer, and that in the absence of a virally encoded E6 protein that mediates p53 degradation this loss of function occurs via somatic mutation. We have examined this hypothesis by investigating the correlation between HPV infection and p53 mutation in a series of primary cervical cancers.

Patients and methods

Tissue samples were obtained from 28 patients undergoing Wertheim's hysterectomy for stage Ib or IIa cervical cancer diagnosed by colposcopically directed punch or minicone biopsy. After removal of the uterus, tissue was taken from the primary tumour and uninvolved vaginal cuff, and frozen rapidly to −70°C. Sections of tumour were stained by haematoxylin and eosin for histological classification. To determine the relative volume of tumour and stromal nuclei in the samples, point counting was done under high magnification with a stratified random sampling technique until 400 nuclei had been sampled.[13] All the tumours contained a majority of tumour cells by pathological examination. This finding was confirmed by counting nuclei in some samples and showing that these samples contained between 65% and 85% of tumour cells; differences in the observed scores were related to the pattern of tumour growth, some tumours growing as solid masses and others as islands infiltrating the stroma.

RESULTS OF ANALYSES OF CERVICAL TUMOURS

Patient	HPV type	Tumour type	p53 sequence	No of 17p alleles (normal/tumour)	
				Probe YNZ22	Probe 144D6
96	18	A	wt	1/1	1/1
240	16	S	–	2/2	2/2
255	16	S	wt	–	–
299	18	S	wt	1/1	2/2
312	16	S	wt	1/1	2/2
314	16	S	wt	–	–
325	Negative	S	M*	–	–
343	Negative	S	M*	1/1	2/2
345	16	S	wt	–	–
350	16	S	wt	2/2	1/1
353	16	S	wt	–	–
355	16	S	wt	–	–
366	16	A	wt	–	–
368	16	S	wt	–	–
372	16	S	wt	–	–
385	Negative	S	M*	2/2	2/2
387	16	A	–	2/2	2/2
389	16	S	–	2/2	2/2
391	16	S	wt	2/2	2/2
393	16	S	wt	2/2	1/1
397	18	A	wt	2/2	2/2
403	16	S	wt	–	–
405	16	S	wt	–	–
407	16	S	wt	1/1	2/2
409	16	S	wt	2/2	2/2
411	18	S	–	2/2	2/2
413	18	S	wt	2/2	1/1
417	16	A	wt	1/1	2/2

S = squamous-cell carcinoma, A = adenocarcinoma, wt = wild-type, M = mutant, – = not analysed.
*PCR sequencing revealed both wild-type and mutant alleles

High-molecular-weight DNA isolation, polymerase chain reaction (PCR) analysis for HPV types, and chromosome 17p restriction fragment length polymorphism (RFLP) analysis of cervical cancers were done as described previously.[12] Asymmetric PCR sequencing was done essentially as described previously,[14] but with some methodological modifications and additional primers for both PCR and sequencing to allow sequencing of the entire coding region of p53. Details of methods and sequences are available from the authors. Briefly, PCR reactions included 1 μg of genomic DNA in 100 μl reaction volumes containing 50 nmol/l of each primer and 2 units of *Taq* polymerase (Promega, Madison, Wisconsin, USA). When sufficient tumour material was available, total cellular RNA was isolated from snap-frozen tissue by guanidinium/phenol extraction and reverse transcription with 2 μg of total RNA.[14] Asymmetrically amplified single-strand DNA was purified and sequenced with T7 DNA polymerase (Promega) and ^{35}S-dATP. Amplified nucleic acids were resolved on 6% polyacrylamide gels. Each tumour DNA was sequenced throughout the entire p53 coding sequence. Proposed mutations were confirmed on both strands. When a p53 mutation was identified, the p53 coding sequences from normal vaginal tissue were also sequenced to confirm that the mutations were specific to the tumour tissue.

Results

Results for each tumour are summarised in the table. Cancer DNAs were typed for HPV by Southern blotting, and those negative by Southern blotting were subjected to PCR analysis. Of the 28 tumours, 5 were adenocarcinomas, of which 3 were HPV16 positive and 2 were HPV18 positive. The remaining 23 tumours were of the squamous-cell type (17 HPV16 positive and 3 HPV18 positive). 3 squamous-cell cancers negative for HPV16 and HPV18 by Southern blot and PCR were tested for HPV types 6b, 11, 31, and 33 by type-specific PCR analysis and found to be negative for these viruses. Since we tested for the types of genital HPV commonly found in European cervical cancers, we assume that HPV-negative tumours arose via a rare HPV-independent mechanism.

DNA from paired normal and tumour tissue specimens from 17 patients was subjected to RFLP studies of 17p allelic deletion by digestion with *Pst*I and Southern blot hybridisation analysis with the probes p144D6 and pYNZ22. DNA from more than 85% of normal tissue specimens shows two alleles with at least one of these probes.[15] One patient was homozygous for both loci and hence noninformative. Normal tissue from the remaining 16 patients showed two alleles with one or both probes, and no evidence for 17p deletion was found in any of the corresponding tumours (table). Contamination of tumour sample with normal tissue was slight since no evidence for allelic imbalance was seen. Representative blots are shown in fig 1.

The entire p53 coding sequence of 24 tumours was determined by asymmetric PCR with either genomic DNA or reverse-transcribed total RNA as the PCR substrate. PCR was done with primers derived from the published cDNA sequence[16] or, when genomic DNA was used, p53 intron sequences (L. Crawford, personal communication). Exons 2–10 were sequenced in their entirety with intron-derived and exon-derived sequencing primers. In each of the 21 HPV-positive tumours, the p53 coding sequences

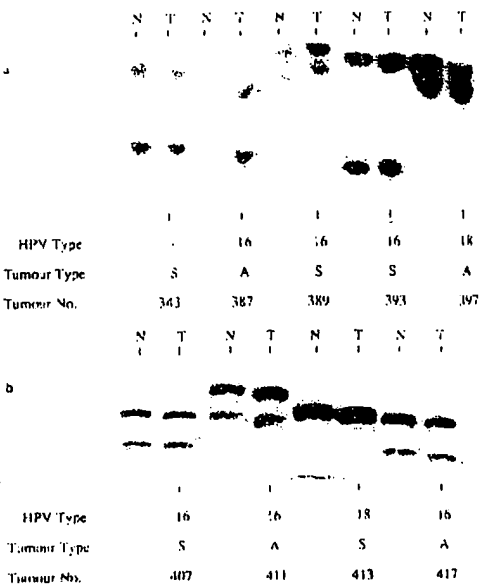

Fig 1—RFLP analysis of 17p alleles in cervical tumours (T) and corresponding normal tissue (N).

Tumours were either squamous-cell cancers (S) or adenocarcinomas (A). HPV type is indicated. DNAs were digested with *PstI*, resolved on 0·8% agarose gels, and subjected to Southern blot hybridisation analysis with either p144D6 (panel a) or pYNZ22 (panel b) probes.

were wild-type. By contrast, the 3 HPV-negative tumours contained somatic p53 point mutations. Tumour 325 had a mutation identical to that seen in the C33a cell line—namely, CGT→TGT at codon 273, converting arginine to cysteine. Tumour 343 had a codon 249 mutation, AGG→AGT, converting arginine to serine, and tumour 385 had a codon 240 mutation, AGT→ATT, converting

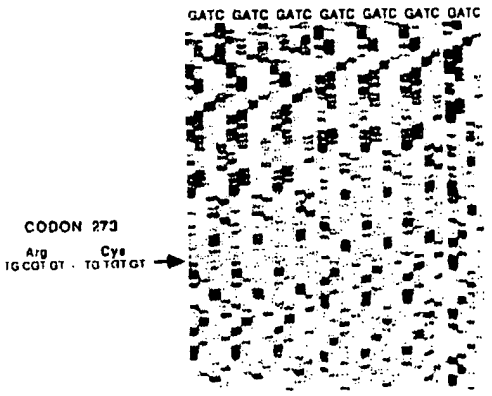

Fig 2—Analysis of p53 coding sequences in cervical tumours and normal tissue.

Single-stranded sequencing templates were generated by asymmetric PCR and sequenced with T7 DNA polymerase (see text). The p53 sequence of exon 8 in tumour 325 is shown in the four tracks on the left, with the position of the mutant T residue arrowed. Subsequent tracks show wild-type sequence in normal tissues from the same patient, followed by wild-type sequence in 5 HPV-positive tumours.

serine to isoleucine. In all three cases, the mutation appeared to be heterozygous, a wild-type allele being present and represented on the sequencing gels of asymmetrically copied genomic DNA (fig 2). Although the wild-type allele may represent p53 sequences in normal tissue present as a contaminant, heterozygosity of the p53 mutation is consistent with the lack of allelic deletion and imbalance seen in the 2 HPV-negative tumours for which sufficient DNA was available for RFLP analysis. The 3 somatic p53 point mutations were confirmed as genuine by sequencing both strands of DNA from two separate PCR reactions, and were also shown to be specific to the cervical tumour because normal vaginal tissue from the same patients retained wild-type p53 genomic sequences (fig 2).

Discussion

Our finding of HPV sequences in 25 of 28 primary cervical tumours is consistent with other reports of an 80–90% prevalence of transforming HPV subtypes in these tumours,[9,17,18] and confirms that in the UK most primary cervical tumours are likely to be HPV positive. Because the inverse relation between p53 mutation and HPV positivity was first demonstrated in eight cervical carcinoma cell lines,[11] it might be argued that the presence of such mutations in two HPV-negative lines was required for their adaptation for growth in culture. Our study is the first to show that this relation is also true for primary cervical cancer and as such provides indirect but strong evidence of the role of HPV in the aetiology of most of these tumours. Our series is not large enough for statistical analysis, but when our data are combined with those reported previously for anal cancer (6 HPV-positive cases, all p53 wild-type; 3 HPV-negative, all with p53 point missense mutations)[12] the inverse relation between HPV positivity and p53 mutation is highly significant ($p < 0{\cdot}0001$, Fisher's exact test).

Whereas p53 mutation, often accompanied by allelic loss at 17p, is common in epithelial cancers,[19–21] it is apparent that in primary anogenital cancers containing HPV sequences such p53 abnormalities are rare. Moreover, in all primary anogenital cancers we have analysed, detectable p53 mutation is confined to HPV-negative tumours. Asymmetric PCR sequencing provides an average measure of p53 sequences in the tumour tissue analysed. Therefore, it is possible that there are small populations of cells within the tumour that contain mutant p53 alleles at an insufficiently high frequency to be detected on the sequencing gels. This possibility could be addressed by sequencing many plasmid clones of p53 PCR products, although this approach is itself complicated by the high frequency of point mutation generated by the amplification procedure, a problem circumvented by asymmetric PCR sequencing. Nevertheless, the absence of evidence of mutant alleles in our study implies that most p53 sequences in HPV-positive tumours are wild-type—ie, clonal p53 mutation is not a requirement for the pathogenesis of HPV-positive primary anogenital cancers.

p53 mutations in HPV-negative tumours have been described previously in other human cancers. G→T transversions resulting in a serine for arginine substitution at position 249 are common in African[22] and Chinese[23] hepatocellular carcinomas, and it is possible that the specificity of mutation seen in these cancers is the result of exposure to the mutagen aflatoxin B_1. The same mutation has also been detected in p53 from a lung cancer.[24] When considering all types of human cancer, 13% of p53 mutations occur at codon 273, making this the most

common site of mutation.[25] The cysteine for arginine substitution at position 273 that we describe has been identified previously in an HPV-negative cervical cancer cell line.[11] Mutations at codon 240 have been detected less often, although a substitution at this position has been reported in a primary colorectal tumour.[26] The mutual exclusivity of HPV infection and p53 mutation in primary cancers implies that there is little selective pressure for p53 mutation in anogenital carcinogenesis when p53 wild-type function is inhibited by a virally encoded protein; furthermore, it provides indirect but strong support for a critical role in the aetiology of anogenital cancers of the ability of the HPV16 E6 protein to target wild-type p53 protein for degradation,[7,8] a process that has been demonstrated only in vitro.

Our data have important clinical implications in the context of the apparently worse prognosis of HPV-negative cervical tumours.[9,10] The predicted result of E6 expression in HPV-positive cancers would be loss of wild-type p53 function resulting from loss of protein in the cell. Similarly, a point mutation of one p53 allele and the resultant expression of an altered p53 protein may lead to loss of wild-type function by a dominant negative mechanism in which the mutant protein binds to normal p53 and inactivates it.[27] However, such mutations may also have other consequences in addition to loss of wild-type p53 function. It is clear that mutant p53 proteins can lose transformation-suppressor activity and gain positive transforming function.[14] It is interesting, therefore, that p53 clones expressing the codon 273 cysteine for arginine mutation and the codon 249 arginine to serine mutation show potent in-vitro transforming activity (unpublished observation). Thus, it is possible that the mutant p53 proteins potentially expressed in the HPV-negative cervical cancers are contributing to the more aggressive phenotype shown by these tumours. Prospective studies of the HPV and p53 status of a large number of cervical tumours will be required to determine whether this hypothesis is correct.

We thank Dr Lionel Crawford for providing unpublished p53 intron sequences, Dr P. Farrell for reviewing the text, and Mr Phillip Masterson and Ms Kathy Mills for technical assistance.

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From The Lancet

Metropolitan migrations

A physician and a medical teacher in a leading metropolitan school does admirable service when he leaves for a moment the bedside and study of individual cases, and summons his class to consider the health bearings of the civilisation in the midst of which we are living. This is what Dr Vivian Poore has been doing in the admirable lecture on the Concentration of Population in Cities, published in the last two numbers of *The Lancet*. When a hospital physician of clinical reputation and industry bids us consider for an hour the bearings of the steadily increasing tendency of population to leave the country and to concentrate in towns our attention is naturally arrested; and as he marshals his facts before us, we are bound to admit that he is stating a problem demanding the serious attention not only of medical men, but of statesmen. We cannot too warmly recommend all public men, all those who have the power by word, or pen, or law, or example to influence public opinion and the movement of population, to read this extremely interesting lecture. For, after all, it is to statesmen that we must largely look for help in diffusing population. It is to be hoped that the development of local government will have some effect in this direction. Anything which supplies men with local function, local interest, and local importance tends powerfully to fix them in their locality. The centripetal tendency of the population is appalling. London is becoming more and more overgrown and the country correspondingly deserted. It is the same in the United States. Life is lived in a crowd, and in a hurry, and, in London at all events, too often in a fog.

(May 28, 1892)

ITEM 6

[CANCER RESEARCH 53, 2231-2234, May 15, 1993]

Advances in Brief

p53 Mutation and *MDM2* Amplification in Human Soft Tissue Sarcomas[1]

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Abstract

The *p53* and *MDM2* genes were analyzed in 24 human soft tissue sarcomas (11 malignant fibrous histiocytomas and 13 liposarcomas). Alterations of *p53*, consisting of point mutations, deletions, or overexpression, were detected in one-third (8 of 24) of the sarcomas. *MDM2* gene amplification was detected in another 8 tumors, but no tumor contained an alteration of both genes. Monoclonal antibodies reactive with the human *MDM2* gene product were developed, and immunohistochemical analysis revealed nuclear localization and overexpression of MDM2 in those tumors with amplified *MDM2* genes. These data support the hypothesis that *p53* and *MDM2* genetic alterations are alternative mechanisms for inactivating the same regulatory pathway for suppressing cell growth.

Introduction

The *p53* gene is mutated in many, but not all, human malignancies (1). What is the role of p53, if any, in those tumors without evident *p53* mutation? Three possibilities can be considered. First, *p53* mutations could exist but be undetectable by standard methods (*e.g.*, mutations in introns which affect expression). Second, mutations could exist in other genes that interact with *p53* or are downstream of *p53* and result in an identical physiological defect within the cell. Third, mutations in other genes, totally unrelated to *p53*, could occur in some tumors, resulting in a transformation process that is qualitatively different from that occurring in *p53*-mutant cells.

In the current study, we sought to address aspects of this important question in human soft tissue sarcomas. Previous analyses of these tumors have revealed that *p53* abnormalities are relatively frequent (30–60%) (2–6). Moreover, a gene (*MDM2*), the product of which binds to p53 (7), has been shown to be amplified in a subset of such tumors (8). If a major effect of *MDM2* amplification were to inactivate the *p53* gene product, one would expect that those tumors with *MDM2* gene amplification would be devoid of *p53* mutations and vice versa—double mutations in both *p53* and *MDM2* would be redundant for the neoplastic process and would provide no selective advantage over that conferred by mutation in only one of the two genes. This hypothesis was tested in the current study using genetic and immunohistochemical methods.

Materials and Methods

Tumors. Ten primary malignant fibrous histiocytomas and 13 primary liposarcomas from 23 patients were frozen immediately after surgery. The OsA-CL cell line was derived from a sarcoma that occurred in bone (9) but had histological features characteristic of a malignant fibrous histiocytoma and is considered here to be a tumor of the latter type. Most of these tumors have previously been evaluated for *MDM2* gene amplification (8) but not for expression of *MDM2* or alteration of *p53*.

DNA Analysis. *p53* exons 5, 6, 7, and 8 were amplified using the polymerase chain reaction as previously described (10) except that the 5' primer contained an artificial *Bam*HI site and the 3' primer contained an artificial *Eco*RI site. The resultant 1.8-kilobase PCR[3] product was digested with *Eco*RI and *Bam*HI, gel purified, and cloned between the *Bam*HI and *Eco*RI sites of pBluescript (Stratagene). DNA purified from pools of at least 100 clones was sequenced with primers specific for each exon (10). Southern blot analysis was performed by digesting 4 μg of DNA with *Eco*RI, separating the fragments by gel electrophoresis, and transferring them to nylon filters. The DNA on filters was then sequentially hybridized with probes for *MDM2* (clone C14-2; Ref. 8), for *p53* (1.8-kilobase complementary DNA containing all coding exons; Ref. 11), and for control sequences on chromosome 17p12 (EW503; Ref. 12). Probe labeling and hybridization were performed as previously described (8, 13).

Monoclonal Antibody Production. Female (BALB/C × C57BL/6) F1 mice were immunized and boosted by i.p. injection of purified GST-MDM2 fusion protein in Ribi adjuvant (Ribi Immunochem Research, Inc.). The fusion protein, containing amino acids 27 to 168 of MDM2, was expressed in *Escherichia coli* and purified using glutathione Sepharose. Hybridomas were produced as described (14, 15), except that test bleeds and hybridomas were screened for anti-MDM2 reactivity using trpE-MDM2 and purified GST-MDM2. Two hybridomas were isolated which appeared to react specifically with MDM2. One of them, mAb IF-2, was found to be particularly useful since it was reactive with human MDM2 in Western blots, immunoprecipitation, and immunohistochemical assays (on frozen, but not paraffin, sections).

Immunohistochemistry. Frozen sections of 6-μm thickness were fixed with Histochoice (Amresco) for 10 min following air drying. After blocking endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the section was incubated with goat serum for 30 min at room temperature and then incubated with antibodies diluted in goat serum for 2 h at room temperature in a humidified chamber. The antibodies used were IF-2 (specific for MDM2, used at 5 μg/ml), 1801 (specific for p53, used at 0.5 μg/ml; Oncogene Science), and CF-11 (same Ig isotype as IF-2, generated against an irrelevant protein, Ref. 15, and used at 10 μg/ml as a negative control; no staining was observed in sections adjacent to those shown in Fig. 5). Following washing with phosphate-buffered saline, the sections were incubated with biotinylated goat anti-mouse Ig and developed with a horseradish peroxidase system (Vectastain Elite, Vector Labs).

Results

To search for gross alterations of the *p53* gene, DNA from each of the 24 sarcomas was digested with *Eco*RI, and Southern blot analysis was performed with the p53 probe. In three of the tumors, a deletion of the normal-sized 18-kilobase fragment was observed, as evidenced by the very faint bands at this position (Fig. 1, *Lanes 1, 4,* and *6*). Ethidium bromide staining demonstrated that all lanes contained equal quantities of undegraded DNA (not shown). To demonstrate the specificity of the *p53* deletions, the blots were rehybridized with another probe for chromosome 17p. Fig. 1 (*bottom*) shows that the EW503 probe, detecting sequences on chromosome 17p near *p53* (12), efficiently hybridized to a 3.3-kilobase DNA fragment from all tumors,

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[2] To whom requests for reprints should be addressed, at The Johns Hopkins Oncology Center, 424 North Bond Street, Baltimore, MD 21231.

[3] The abbreviations used are: PCR, polymerase chain reaction; mAb, monoclonal antibody; HPV, human papillomavirus.

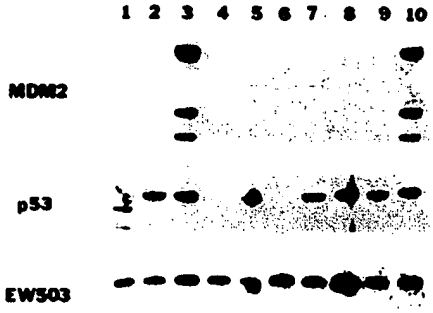

Fig. 1. Southern blot analysis of the *MDM2* and *p53* genes in human sarcomas. Southern blotting was performed using probes for p53, MDM2, and EW503, as described in "Materials and Methods." The hybridizations were sequentially performed with the same blot. *Lanes 1* to *10*, tumors 1 to 10, respectively (Table 1). The *MDM2* fragments migrated at 8, 4, and 3 kilobases, the *p53* fragment at 18 kilobases, and the EW503 fragment at 3.3 kilobases.

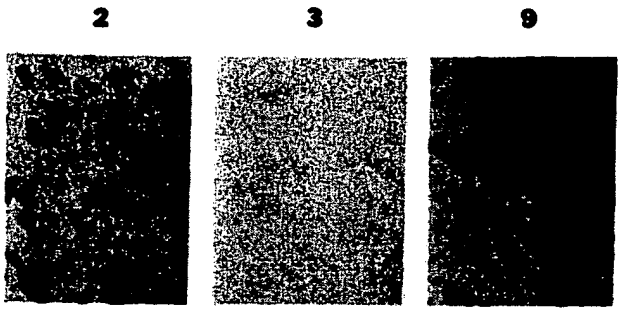

Fig. 2. *p53* expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the mAb 1801 antibody specific for p53 as described in "Materials and Methods." Tumors 2 and 9 showed overexpression of *p53*, while no staining was observed in tumor 3.

including tumors 1, 4, and 6. Of the three tumors with deletion, two showed a total absence of signal (*Lanes 4* and *6*), while one showed two smaller-size bands reactive with the p53 probe (*Lane 1*). These bands were not the result of contamination with plasmid DNA, as shown by the absence of hybridization to a radiolabeled probe containing only vector sequences (not shown). Thus, deletion of *p53* in tumor 1 was associated with at least one intragenic rearrangement, whereas the deletions observed in tumors 4 and 6 were the result of rearrangements the borders of which were outside the region detected by the p53 probe.

Fig. 1 also shows examples of *MDM2* amplification, noted in 8 of the tumors studied (Fig. 1, *Lanes 3* and *10*; Table 1). Each of these 8 tumors contained at least 10 copies of the *MDM2* gene/cell. The p53 and EW503 probes served as controls for DNA loading and transfer. No rearrangements were noted in tumors with or without amplification, since only the expected fragments of 8, 4, and 3 kilobases were observed. Longer exposures revealed the same size fragments in the tumors without amplification of *MDM2* (not shown).

To detect subtle sequence alterations of *p53*, exons 5–8 were sequenced. These exons have been shown to harbor most of the point mutations observed in human tumors (reviewed in Ref. 1). In each case, a PCR product containing exons 5–8 was cloned, and a pool of at least 100 clones was sequenced. Four sarcomas contained point mutations demonstrable in this assay. Two of these (tumors 2 and 12) contained missense mutations; one tumor (tumor 15) contained a nonsense mutation; and another (tumor 18) contained a mutation altering a consensus splice site (Table 1). In these four cases, the signal corresponding to the normal nucleotide sequence of *p53* was weaker than that of the mutant nucleotide, suggesting that the mutation was accompanied by a loss of the wild-type allele, with the residual signal contributed by nonneoplastic cells within the tumor (not shown). In each of the four cases, an independent PCR and sequencing reaction were performed to confirm the mutation.

For seven of the tumors used in this study, frozen samples were available for immunohistochemical analysis. In five of the tumors, little or no reactivity with the p53-specific antibody was observed, a result consistent with the absence of *p53* mutation. In contrast, two tumors (tumors 2 and 9) showed strong nuclear staining with the

Table 1 *Profile of tumors and mutations*

Tumor no.	Tumor ID	Type[a]	*MDM2* amplification[b]	*p53* alteration[c]	Over expression[d]
1	M-2	MFH	Absent	Deletion/rearrangement	None
2	M-5	MFH	Absent	CGC-CUC mutation; Arg(158)-His	*p53*
3	M-7	MFH	Present	None observed	*MDM2*
4	M-8	MFH	Absent	Deletion	None
5	M-14	MFH	Absent	None observed	NT
6	M-15	MFH	Absent	Deletion	NT
7	M-16	MFH	Absent	None observed	None
8	M-17	MFH	Absent	None observed	NT
9	M-18	MFH	Absent	Overexpressed	*p53*
10	M-20	MFH	Present	None observed	*MDM2*
11	L-5	Liposarcoma	Absent	None observed	NT
12	L-7	Liposarcoma	Absent	AAC-AGC mutation; Asn(239)-Ser	NT
13	L-9	Liposarcoma	Present	None observed	NT
14	L-11	Liposarcoma	Absent	None observed	NT
15	KL5B	Liposarcoma	Absent	CAG-UAG mutation; Gln(144)-stop	NT
16	KL7	Liposarcoma	Present	None observed	NT
17	KL10	Liposarcoma	Absent	None observed	NT
18	KL11	Liposarcoma	Absent	GGT-GAT mutation; exon 5 splice donor site	NT
19	KL12	Liposarcoma	Absent	None observed	NT
20	KL28	Liposarcoma	Present	None observed	NT
21	KL30	Liposarcoma	Present	None observed	NT
22	S189	Liposarcoma	Present	None observed	NT
23	S131B	Liposarcoma	Absent	None observed	NT
24	OSA-CL	MFH	Present	None observed	*MDM2*

[a] MFH, malignant fibrous histiocytoma.
[b] As assessed by Southern blot.
[c] As assessed by Southern blot, sequencing of exons 5–8, or immunohistochemical analysis.
[d] As assessed by immunohistochemical analysis. NT, not tested.

antibody (Fig. 2). In tumor 2, this reactivity was expected because the tumor contained a missense mutation (Table 1). In tumor 9, no mutation in exons 5–8 was detected, and the mutation giving rise to the overexpression was presumably outside the region sequenced. There is ample precedent for occasional *p53* mutations outside exons 5–8 in other tumor types (1, 16).

To evaluate *MDM2* expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. Of several antibodies tested, mAb IF-2 was the most useful, since it detected MDM2 in several assays (see "Materials and Methods"). For initial testing, we compared proteins derived from OsA-CL, a sarcoma cell line with *MDM2* amplification but without *p53* mutation (Table 1) and proteins from SW480, a colorectal cancer cell line with *p53* mutation (11) but without *MDM2* amplification (data not shown). Fig. 3 shows that the mAb IF-2 detected an intense M_r 90,000 band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense M_r 90,000 band in SW480 extracts. We could not distinguish whether the low-molecular-weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in *MDM2* gene copy number in these two lines. Conversely, the M_r 53,000 signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL, consistent with the presence of a mutated *p53* in SW480 (Fig. 3).

Cells grown on coverslips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb, and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 4). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (17) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186 (8). Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 4), with the relative intensities consistent with the Western blot results (Fig. 3).

The IF-2 mAb was then used to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors 3 and 10) stained strongly (Fig. 5). Both of these tumors contained *MDM2* gene amplification (Table 1). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 5).

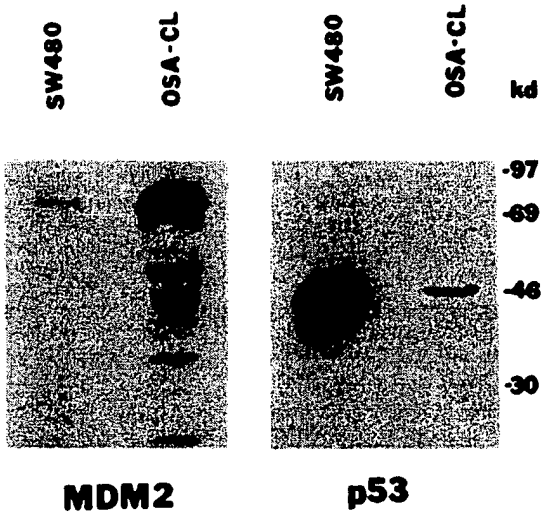

Fig. 3. Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty μg of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis, as described in "Materials and Methods." The position of molecular weight markers, in kilodaltons, is given on the *right*.

Discussion

The results of this study show that at least two-thirds of the soft tissue sarcomas analyzed contained alterations of *p53* or *MDM2*. Importantly, tumors contained either a *p53* alteration or an *MDM2* alteration, but not both. This distribution was significant ($P < 0.013$, χ^2) and supports the hypothesis that the major effect of *MDM2* amplification is identical to that resulting from *p53* mutation; otherwise, *p53* gene mutations would be expected to occur at equal frequencies in tumors with or without *MDM2* amplification.

This interpretation is consistent with biochemical and physiological data documenting *p53-MDM2* interaction. *MDM2* is an oncogene (18, 19) which binds to *p53 in vivo* and *in vitro* (7, 8). *p53* is thought to function by transcriptionally activating target genes through an acidic activation domain located at codons 20–42 (reviewed in Ref. 20). These target genes contain two copies of a 10-base pair *p53*-specific DNA binding motif within their controlling regions. Overexpression of *MDM2* has been shown to inhibit the ability of *p53* to stimulate expression from such target genes (7, 21). Moreover, it has recently been shown that this inhibition is likely to result from *MDM2* binding directly to the acidic activation domain of *p53*, concealing it from the transcriptional machinery (21). Thus, the biochemical data are in accord with the results presented here; one would expect that either *p53* or *MDM2* would be altered in a given sarcoma but that mutations in both genes would be functionally redundant and should not be observed.

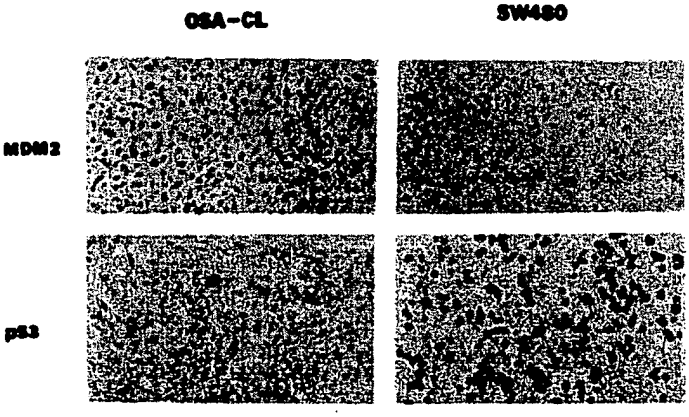

Fig. 4. Immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used as described in "Materials and Methods." The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

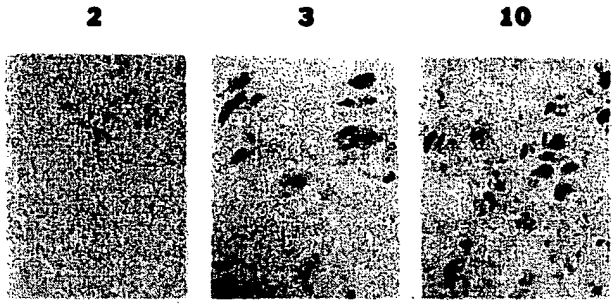

Fig. 5. *MDM2* expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2 as described in "Materials and Methods." Tumors 3 and 10 showed nuclear expression of MDM2, while tumor 2 showed no staining.

The closest analogue to the *MDM2/p53* relationship in sarcomas is provided by *E6/p53* in cervical cancers (22). The E6 oncoprotein encoded by HPV types 16 and 18 can functionally inactivate *p53* (23). Accordingly, it has been reported that cervical cancers with HPV infection infrequently contain *p53* gene mutations (24). However, there have been some exceptions to this paradigm, since tumors containing both HPV sequences and *p53* mutations have been discovered (25). It remains to be seen whether similar exceptions regarding *MDM2* and *p53* will be found as additional sarcomas are analyzed, but the present data suggest with high statistical significance that alterations of these two genes are mutually exclusive.

Finally, what about the soft tissue sarcomas (one-third of the total) without evident *p53* mutations or *MDM2* gene amplification? It is possible that more detailed analyses of such tumors will reveal other alterations of *p53* or *MDM2*, such as point mutations outside exons 5–8 in *p53* or increased expression of *MDM2* in the absence of amplification. Alternatively, some of these tumors might progress through genetic events that involve a totally different pathway. It will be of interest in the future to correlate histopathology, disease course, and response to therapy in sarcomas with and without alterations of *p53* or *MDM2*. Additionally, further examination of such tumors might allow the discovery of genes other than *MDM2* that can functionally inactivate *p53* or its downstream effectors.

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ITEM 7

Amplification of a gene encoding a p53-associated protein in human sarcomas

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DESPITE extensive data linking mutations in the p53 gene to human tumorigenesis[1], little is known about the cellular regulators and mediators of p53 function. MDM2 is a strong candidate for one such cellular protein; the *MDM2* gene was originally identified by virtue of its amplification in a spontaneously transformed derivative of mouse BALB/c cells[2] and the MDM2 protein subsequently shown to bind to p53 in rat cells transfected with p53 genes[3,4]. To determine whether MDM2 plays a role in human cancer, we have cloned the human *MDM2* gene. Here we show that recombinant-derived human MDM2 protein binds human p53 *in vitro*, and we use *MDM2* clones to localize the human *MDM2* gene to chromosome 12q13–14. Because this chromosomal position appears to be altered in many sarcomas[5–7], we looked for changes in human *MDM2* in such cancers. The gene was amplified in over a third of 47 sarcomas, including common bone and soft tissue forms. These results are consistent with the hypothesis that MDM2 binds to p53, and that amplification of *MDM2* in sarcomas leads to escape from p53-regulated growth control. This mechanism of tumorigenesis parallels that for virally-induced tumours[8,9], in which viral oncogene products bind to and functionally inactivate p53.

To obtain human complementary DNA clones, a murine *MDM2* cDNA probe was used to initiate cDNA walking in a human library (see legend to Fig. 1). Sequence analysis of 25 clones revealed several cDNA forms indicative of alternative splicing. The predominant human form is compared with its murine counterpart in Fig. 1. There was an open reading frame extending from the 5′ end of the human cDNA sequence to nucleotide 1,784. Although this signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between human and mouse *MDM2* declined dramatically upstream of nucleotide 312. Second, an inverse polymerase chain reaction (PCR) was used in an attempt to acquire additional upstream cDNA sequence[10]. The 5′ ends of the PCR-derived clones were very similar (within 12 base pairs) to the 5′ ends of clones obtained from the cDNA library, indicating that the 5′ end of the human *MDM2* sequence shown in Fig. 1 may represent the 5′ end of the transcript. Third, *in vitro* translation of the sequence shown in Fig. 1, beginning with the methionine encoded by the ATG at position 312, generated a protein similar in size to that observed in human cells (see below).

Comparison of the human and mouse *MDM2* coding regions showed that they were 80.3% identical and shared a basic nuclear localization signal at codons 181 to 185 (ref. 11), several casein kinase II serine-phosphorylation sites[12], an acidic activation domain at codons 223 to 274 (ref. 13), and two metal-binding sites at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA-binding domains[14].

To determine whether the human MDM2 protein could bind to human p53 protein *in vitro*, a human *MDM2* expression vector was constructed from the cDNA clones (see legend to Fig. 2). RNA transcribed from this vector using T7 RNA polymerase was used to program a rabbit reticulocyte lysate. Although the predicted size of the protein generated from the construct was only 55.2K (M_r 55,200, extending from the methionine at nucleotide 312 to nucleotide 1,784), protein translated *in vitro* migrated at ~90 K. The MDM2 protein was not immunoprecipitated with antibodies against either the C-terminal or N-terminal regions of p53 (Fig. 2, lanes 2 and 3). But when *in vitro*-translated human p53 was mixed with the human *MDM2* translation product, the anti-p53 antibodies precipitated MDM2 protein with p53 (Fig. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility (MCC[15]) was mixed with p53 and there was no coprecipitation (Fig. 2, lanes 8 and 9). When an *in vitro*-translated His-175 mutant form of p53 was mixed with human MDM2 protein, a similar coprecipitation of MDM2 and p53 proteins was also observed (data not shown).

Polyclonal rabbit antibodies were raised against an *Escherichia coli*-produced human MDM2-glutathione S-transferase fusion protein. The anti-MDM2 antibodies immunoprecipitated p53 when mixed with MDM2 protein (Fig. 2, lane 15) but failed to precipitate p53 alone (Fig. 2, lane 13).

To establish the chromosomal localization of human *MDM2*, somatic cell hybrids were screened, and a human–hamster hybrid containing only human chromosome 12 hybridized to the human *MDM2* probe. Screening of hybrids containing portions of chromosome 12 (ref. 16) with the same probe narrowed the localization to chromosome 12q13–14. Because this region of chromosome 12 is often aberrant in human sarcomas[5–7], southern blot analysis to evaluate whether *MDM2* was genetically altered in such cancers. We found a striking amplification of *MDM2* sequences in several of these tumours (see examples in Fig. 3, lanes 2, 3 and 5). Of 47 sarcomas analysed, 17 showed a 5–50-fold *MDM2* amplification. These tumours included 7 of 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas, 3 of 11 osteosarcomas, and 0 of 1 rhabdomyosarcoma. Five benign soft tissue tumours (lipomas) and seventy-four carcinomas (colorectal or gastric) were also analysed by Southern blotting and no amplification was seen.

We next determined whether this gene amplification was associated with increased expression. Because of RNA degradation in primary sarcomas, only the cell lines could be productively analysed by northern blotting. In the one available sarcoma cell line with *MDM2* amplification, a single transcript of ~5.5 kilobases (kb) was observed (Fig. 4*a*, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Fig. 4*a*, lane 2) or in a carcinoma cell line (Fig. 4*a*, lane 3). When purified messenger RNA (rather than total RNA) from the carcinoma cell line was used for analysis, a human *MDM2* transcript of 5.5 kb could also be observed (Fig. 4*a*, lane 4). Expression of the *MDM2* RNA in the sarcoma with amplification was estimated to be at least 30-fold higher than that in the other lines examined. This was consistent with results from western blot analysis. A protein of M_r ~90K was expressed at high levels in the sarcoma cell line with *MDM2* amplification (Fig. 4*b*, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Fig. 4*b*, lanes 1, 2 and 4). Five primary sarcomas were also analysed by western blotting. Three primary sarcomas with amplification expressed the same sized protein as that in the sarcoma cell line (Fig. 4*c*, lanes 1–3), but no protein was observed in the two sarcomas without amplification (Fig. 4*c*, lanes 4 and 5).

Our results demonstrate that human MDM2 binds to p53 *in vitro* and is genetically altered in a significant fraction of the most common sarcomas of soft tissue and bone[17,18]. It is important to note, however, that amplifications in human tumours

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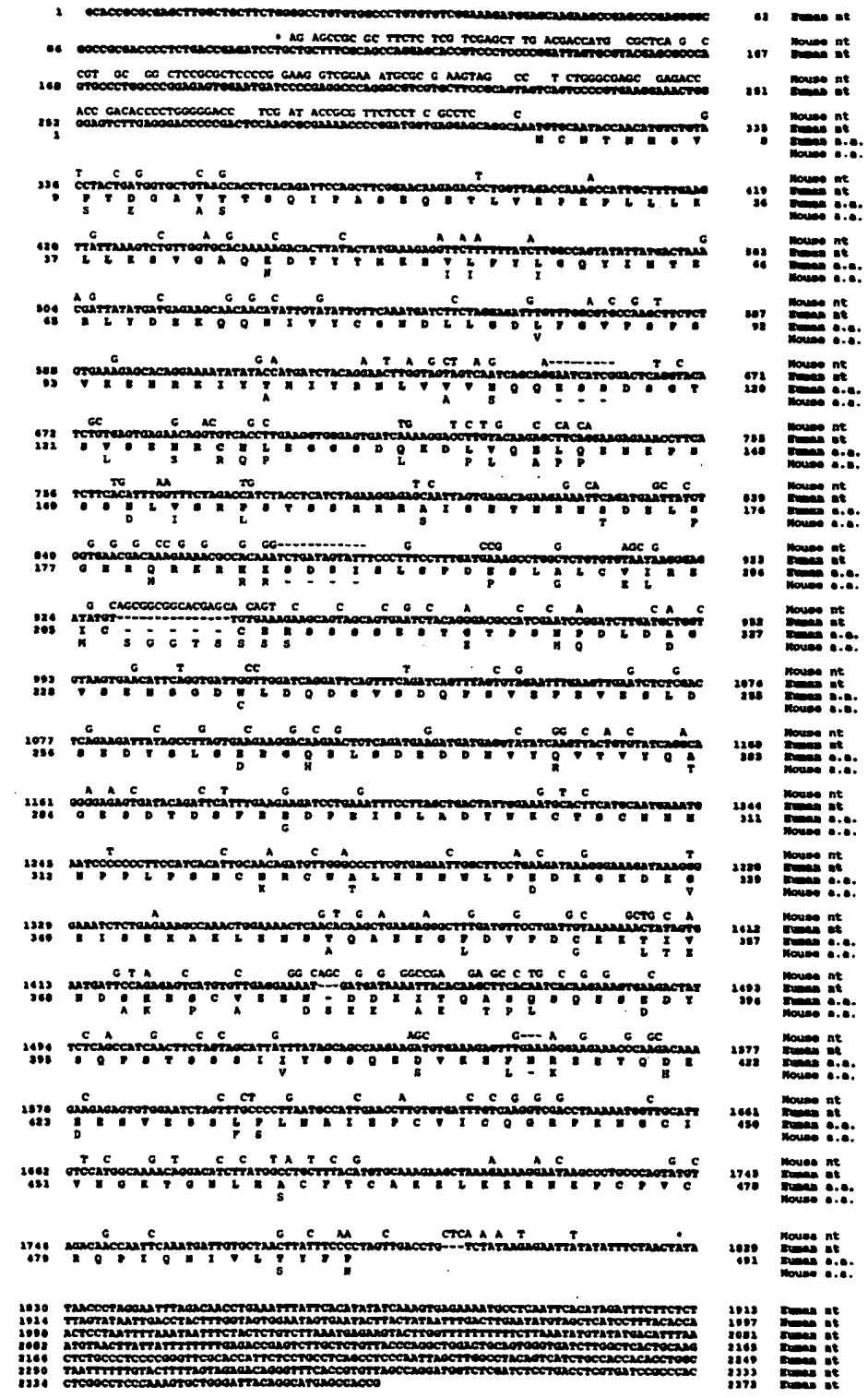

FIG. 1 Human *MDM2* cDNA sequence: human and mouse *MDM2* nucleotide (nt) and amino-acid (a.a.) sequences are compared (single-letter code). The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mouse *MDM2* cDNA[2]. Dashes indicate insertions.

METHODS. Polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 was used as a template for the production of random hexamer-primed double-stranded cDNA[22]. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged and plated as described[23]. The library was screened initially with a ^{32}P-labelled [24] mouse *MDM2* cDNA probe (nt 259 to 1,508; ref. 2) and then rescreened with a human *MDM2* cDNA clone containing nt 40 to 702. In total, 25 clones were obtained, partially or totally sequenced, and mapped. The sequence shown is representative of the most abundant class and was assembled from three clones: c14-2 (nt 1–949), c89 (nt 467–1,737), and c33 (nt 390–2,372). The 3' end of the untranslated region has not yet been cloned. The 5' end is likely to be at or near nt 1 (see text). The mouse and human amino-acid sequences are compared from the putative translation start site at nt 312 to the conserved stop codon at nt 1,784. This nucleotide sequence has been deposited at the EMBL Data Library, accession number Z12020.

often involve large stretches of the genome, encompassing 300 to 1,000 kb[7,19–21]. Other genes in the *MDM2* amplicon could contribute to, or be responsible for, the growth advantage afforded by the amplification event. Nevertheless, MDM2 is a good candidate for the 'target' of amplification for two reasons. First, MDM2 has oncogenic activity after transfection into NIH3T3 cells[2]. Second, it binds to a protein (p53) with known growth suppressive effects on a wide variety of human tumour types. MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as simian virus 40 T antigen, adenovirus E1B and human papilloma virus E6 (refs. 4, 8, 9). Consistent with this hypothesis, no sarcomas with *MDM2* amplification (of five tested) had any of the p53 gene mutations that occur commonly in other tumours

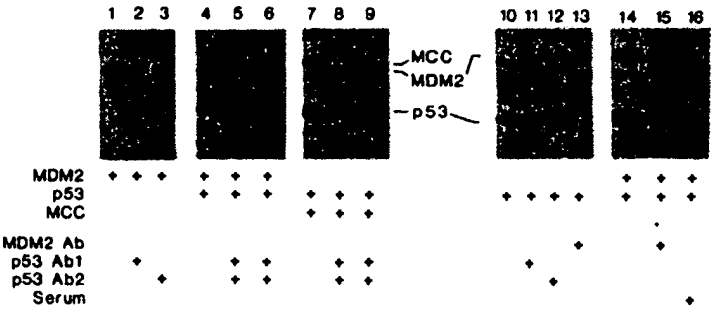

FIG. 2 Coprecipitation of human MDM2 and p53. *In vitro*-translated MDM2, p53 and MCC proteins were mixed as indicated and incubated with p53 Ab1 (monoclonal antibody specific for the C terminus of p53), p53 Ab2 (monoclonal antibody specific for the N terminus of p53), MDM2 Ab (polyclonal rabbit anti-human MDM2 antibodies), or serum (preimmune serum obtained from the rabbit that produced the MDM2 antibody). Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. Bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

METHODS. A human *MDM2* expression vector was constructed in pBluescript SK+(Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Fig. 1 from nt 312 to 2,176. A 42-bp black bettle virus ribosome entry sequence[25] was placed immediately upstream of this *MDM2* sequence in order to obtain high expression. This construct, as well as p53 (ref. 26) and *MCC*[15] constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Lysate (10 μl) containing the three proteins, alone or mixed in pairs, was incubated at 37 °C for 15 min. 1 μg (10 μl) of p53 Ab1 or Ab2 (Oncogene Science) or 5 μl of rabbit serum containing MDM2 antibody or preimmune rabbit serum, were added as indicated. 90 μl RIPA buffer (10 mM Tris, pH 7.5, 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer[3], or binding buffer[26] were then added and the mixtures allowed to incubate at 4 °C for 2 h. The three buffers produced similar results, although the coprecipitation was less efficient in SNNTE buffer (containing 0.5 M NaCl; lanes 5 and 8) than in binding buffer (containing 0.1 M NaCl; lanes 6 and 9). Following addition of 2 mg protein A-Sepharose, the tubes were rotated end-over-end at 4 °C for 1 h. After pelleting and washing, immunoprecipitates were electrophoresed on SDS-polyacrylamide gels and the dried gels autoradiographed in the presence of Enhance (New England Nuclear). Rabbits were immunized with a glutathione *S*-transferase (Pharmacia)–MDM2 fusion protein containing human MDM2 from the region corresponding to nt 390–816.

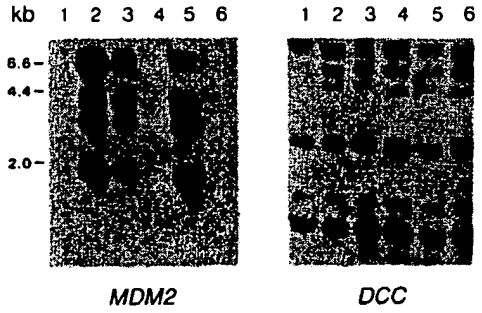

FIG. 3 Amplification of the human *MDM2* gene in sarcomas. DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis and transferred to nylon as described[27]. Filters were then hybridized with a human *MDM2* cDNA fragment probe (nt 1–949; Fig. 1) or to a control probe that identifies fragments of similar size (pDCC 1.65; ref. 28). Hybridization was as previously described[29]. DNA was derived from 5 primary sarcomas (lanes 1–4, 6) and one sarcoma cell line (OsA-CL, lane 5). On longer exposure, the same sized *MDM2* fragments were observed in lanes 1, 4 and 6. DNA fragment sizes are shown on the left in kb.

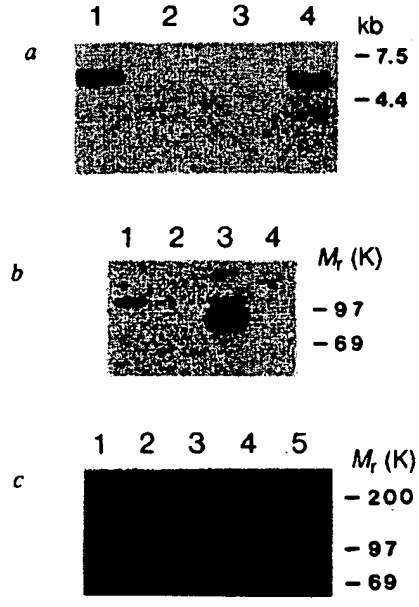

FIG. 4 *MDM2* expression. *a*, Northern blot analysis. RNA was separated by electrophoresis in a MOPS–formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were as described[30]. RNA was hybridized to the *MDM2* fragment described in Fig. 3 legend. Total RNA (10 μg) was derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μg polyadenylated CaCo-2 RNA. RNA sizes are shown on the right in kb. *b*, Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2). *c*, Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with *MDM2* amplifications, and lanes 4 and 5 contain protein from sarcomas without *MDM2* amplification. Western blots using affinity-purified MDM2 antibody were performed with 50 μg protein per lane as described [31], except that the membranes were blocked in 10% non-fat dried milk and 10% goat serum, and secondary antibodies were coupled to horseradish peroxidase to allow chemiluminescent detection (Amersham ECL). MDM2 antibody was affinity-purified with a pATH–MDM2 fusion protein using methods described in ref. 31. Nonspecific reactive proteins of 75, 105 and 170K are seen in all lanes, irrespective of *MDM2* amplification. MDM2 proteins, of M_r 90K, were observed only in the MDM2-amplified tumours. Protein marker sizes are shown on the right.

(unpublished results with T. Tokino and D. Sidransky). The amplification of *MDM2* provides another provocative parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. □

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Wild-type p53 activates transcription *in vitro*

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THE p53 protein is an important determinant in human cancer and regulates the growth of cells in culture[1-3]. It is known to be a sequence-specific DNA-binding protein[4,5] with a powerful activation domain[6-8], but it has not been established whether it regulates transcription directly. Here we show that intact purified wild-type human and murine p53 proteins strongly activate transcription *in vitro*. This activation depends on the ability of p53 to bind to a template bearing a p53-binding sequence. By contrast, tumour-derived mutant p53 proteins cannot activate transcription from the template at all, and when complexed to wild-type p53, these mutants block transcriptional activation by the wild-type protein. Moreover, the simian virus 40 large T antigen inhibits wild-type p53 from activating transcription. Our results support a model in which p53 directly activates transcription but this activity can be inhibited by mutant p53 and SV40 large T antigen through interaction with wild-type p53.

A DNA-binding immunoassay has been used to screen human genomic clones and show that p53 binds specifically to a region upstream of the transcription start site for the human ribosomal gene cluster (RGC)[4]. We have confirmed and extended this observation by DNase I footprinting and shown that addition of immunopurified p53 to a DNA fragment containing the RGC site leads to strong and specific protection of only the RGC region (Fig. 1). All tumour-derived mutant p53 proteins tested failed to protect this sequence (J.B. *et al.*, manuscript in preparation).

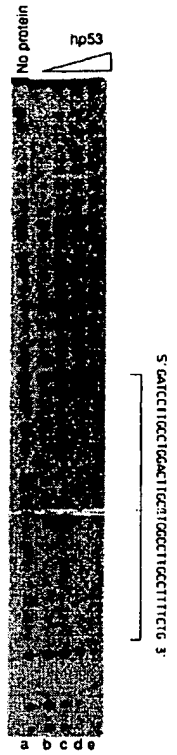

FIG. 1 The p53 protein binds specifically to a site in the human ribosomal gene cluster. DNA binding was assayed in 50-μl volumes containing 40 mM creatine phosphate, pH 7.7, 4 mM ATP, 7 mM $MgCl_2$, bovine serum albumin (0.2 mg ml^{-1}) 0.5 mM dithiothreitol, 10 ng carrier plasmid (pAT153) and 10 fmol of 5' ^{32}P-labelled DNA fragment containing the ribosomal gene cluster (RGC) p53-binding site[4] and either no protein (lane a) or increasing amounts of wild-type human p53 in increments of 15 ng up to 60 ng (lanes b–e). DNase I treatment of mixtures and processing of samples for electrophoresis on 8% polyacrylamide urea gels has been described[5]. Wild-type p53 was immunopurified from SF27 cells expressing a recombinant baculovirus, pEV55hwt, using the monoclonal antibody Pab421 crosslinked to Sepharose A[14].

To determine whether p53 can activate transcription *in vitro*, we used as templates the plasmids fos1wt and fos1mt, which contain the human RGC p53 DNA-binding fragment or a mutated RGC fragment respectively (Fig. 2*a*). Three partially purified fractions from HeLa cell nuclear extracts were used as a source of transcription factors[9]. RNA products were analysed by S1 nuclease digestion using specific probes for each construct. Increasing amounts of p53 stimulated transcription from fos1wt (compare lanes 1-4 with lanes 5-8). These reaction mixtures also included a construct containing an abridged adenovirus major late promoter (pMLS; ref. 10) whose transcription was not significantly affected by p53.

The p53 protein activated transcription from another promoter as well (Fig. 2*b*). Plasmids containing either one or sixteen copies of the RGC site, or one mutant RGC site, inserted adjacent to the polyoma virus early promoter to create Py1wt, Py16wt and Py1mt, respectively, were used as templates in transcription reactions. We found that p53 activated transcription of constructs containing the wild-type RGC (lanes 1-9) but not the mutant RGC (lanes 10-12). Diagrams of the templates and the test probe used in these experiments are shown in Fig. 2*c* with the expected S1 nuclease products.

The high incidence of p53 gene mutations in cancer patients suggests that alteration of the normal function of p53 is an important part of the oncogenic process. Therefore it was of interest to examine whether tumour-derived mutant p53 proteins activate transcription. The mutant p53 proteins we chose are defective in both nonspecific and specific DNA binding[4,5,11], so providing an opportunity to confirm that p53 must bind DNA to activate transcription. We compared the ability of wild-type and two tumour-derived mutant p53 proteins with mutations at either amino acid 175 (His 175) or at amino acid 273 (His 273)

ITEM 8

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Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53

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THE *Mdm2* proto-oncogene was originally identified as one of several genes contained on a mouse double minute chromosome present in a transformed derivative of 3T3 cells[1]. Overexpression of *Mdm2* can immortalize primary cultures of rodent fibroblasts[2]. Human *MDM2* is amplified in 30–40% of sarcomas, and is overexpressed in leukaemic cells[3,4]. The Mdm2 oncoprotein forms a complex with the p53 tumour-suppressor protein and inhibits p53-mediated transregulation of gene expression[5,6]. Because *Mdm2* expression increases in response to p53, Mdm2–p53 binding may autoregulate *Mdm2* expression and modulate the activity of p53 in the cell[7,8]. We have created Mdm2-null and Mdm2/p53-null mice to determine whether Mdm2 possesses developmental functions in addition to the ability to complex with p53, and to investigate the biological role of Mdm2–p53 complex formation in development. Mice deficient for Mdm2 die early in development. In contrast, mice deficient for both Mdm2 and p53 develop normally and are viable. These results suggest that a critical role of Mdm2 in development is the regulation of p53 function.

Mdm2 was cloned from a 129-strain genomic library and the gene structure was characterized to allow the creation of a replacement vector for gene targeting in embryonic stem (ES) cells (Fig. 1*a*). Positive and negative selection of transfected AB2.1 ES cells led to the isolation of one clone which had replaced the 7.1-kilobase (kb) region of *Mdm2* which encodes all putative transcription function motifs of Mdm2 with an *hprt* reporter gene. Injection of the targeted ES cells into host blastocysts gave rise to chimaeric mice which transmitted the mutated *Mdm2* allele (*mdm2*^{nl}) to F_1 progeny (Fig. 1*b*). Intercrosses between mice heterozygous for the targeted allele (*mdm2*^{nl}/+) were performed and the offspring genotyped by Southern ana-

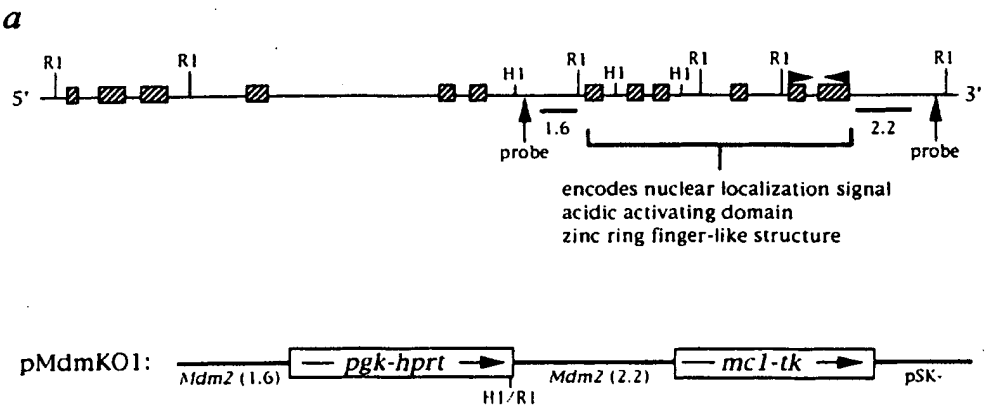

FIG. 1 Gene targeting in *Mdm2*. *a*, Structure of *Mdm2* gene and targeting vector. A replacement targeting vector containing 1.6 kb of 5′ homology and 2.2 kb of 3′ homology was constructed with markers for positive selection (hprt mini-gene)[20] and negative selection (thymidine kinase)[21]. *b*, Southern analysis of targeted ES cell DNA (lane 1), and genomic DNA from a wild-type F_1 progeny mouse (lane 2) and mutant F_1 progeny mouse (lane 3). The presence of a 5.7 kb *Bam*HI band following hybridization with a 5′ probe, and the presence of a 4.7 kb *Eco*RI band following hybridization with a 3′ probe, indicates proper targeting of the *Mdm2* gene in the ES clone by pMdmKO1.
METHODS. The entire *Mdm2* gene was cloned from a 129-strain mouse genomic phage library and characterized by digestion with endonuclease restriction enzymes, including *Bam*HI (HI) and *Eco*RI (RI). A 1.6 kb *Kpn*I–*Eco*RI fragment and a 2.2 kb *Pst*I fragment which flank a 7.1 kb region of *Mdm2* encoding all of the putative transcription factor motifs were used to create the gene replacement vector pMdmKO1. AB2.1 ES cells[20] were electroporated with pMdmKO1 and placed under selection. Mini-Southern analysis[22] of the resulting 500 colonies identified one clone which exhibited proper targeting at the 3′ end. This clone was expanded and analysed by Southern blot hybridizations using a 1.3 kb, *Bam*HI–*Kpn*I 5′ probe and a 350 bp, *Sac*I–*Sca*I 3′ probe. Injection of these ES cells into host blastocysts yielded chimaeric mice that were capable of transmitting the mutated allele to F_1 progeny.

TABLE 1 Analysis of embryos from *mdm2*^{tm}/+ intercrosses

Gestational age	Total number	Reabsorptions	Genotyped	*mdm2*^{tm}/*mdm2*^{tm}
day 9.5	20	6	14	0
day 8.5	25	6	19	0
day 7.5	29	8	21	0

Embryos were collected from female partners of *mdm2*^{tm}/+ intercrosses on day 9.5, 8.5 or 7.5 of gestation. The total number of embryos and the number of embryos undergoing maternal reabsorption are given. Those not undergoing reabsorption were genotyped by PCR using an *Mdm2* primer pair which amplifies a region of genomic DNA between exons 11 and 12 (see horizontal arrows in Fig. 1a). This region of the *Mdm2* gene is deleted following homologous recombination of the gene with the targeting vector. A pair of primers to exon 2 of the *Wnt-5a* gene were present in each reaction to act as a control for the thermocycling reactions and for integrity of the starting template DNA. All of the embryos tested contained DNA that could be amplified using the Mdm2 primers, indicating that none of the embryos were null for *Mdm2* (*mdm2*^{tm}/*mdm2*^{tm}).

lysis of genomic DNA isolated from tail biopsy. Of the 109 progeny obtained from intercrosses of *mdm2*^{tm}/+ mice, 33% were wild-type for *Mdm2* (+/+) and 67% were heterozygous for the targeted *Mdm2* allele (*mdm2*^{tm}/+). No viable *mdm2*^{tm}/*mdm2*^{tm} mice were detected in these crosses, suggesting that absence of functional Mdm2 leads to embryonic lethality. A strategy based on polymerase chain reaction (PCR) was used to determine the gestational time of embryonic lethality of *mdm2*^{tm}/*mdm2*^{tm} mice. Genomic DNA was isolated from the yolk sacs of embryos or from whole embryos collected at various times during development from female partners of *mdm2*^{tm}/+ intercrosses. Coamplification of a region deleted in *mdm2*^{tm} and of a control region in *Wnt-5a* was performed to detect the presence of *mdm2*^{tm}/*mdm2*^{tm} embryos. The results indicate that *mdm2*^{tm}/*mdm2*^{tm} embryos die before day 7.5 of gestation (Table 1). The large percentage (28%) of embryos found to be in the latter stages of reabsorption on day 7.5 suggests that embryonic demise occurs after implantation of the embryo in the wall of the uterus at day 4.5 but before day 7.5 of gestation.

To examine the developmental defect in Mdm2-deficient embryos, histological analysis of embryos collected from female partners of *mdm2*^{tm}/+ intercrosses was performed. Approximately one-quarter (5 of 19) of the embryos examined at day 6.5 of gestation exhibited an abnormal embryonic architecture (Fig. 2). Very little embryonic material was present in the maternal decidua, and the most developed embryo had not progressed significantly beyond day 5.5 of gestation. The time of the developmental block in these presumptive mutants coincides with the sudden increase in cell cycle rate that occurs at day 5.5–6.0 of mouse development[9].

In situ hybridization of wild-type day-7.5 embryo sections indicates that *Mdm2* is ubiquitously expressed in the developing embryo (data not shown). On day 8.5 of development, *p53* is also expressed ubiquitously[10]. To confirm that both *Mdm2* and *p53* are expressed on day 6.0–6.5 of gestation, RNA was isolated from wild-type egg cylinder stage embryos and reverse transcribed into complementary DNA. PCR amplification using oligonucleotide primers for *Mdm2* and *p53* indicates that both transcripts are present when the presumptive mutants die (Fig. 3*a*). These findings are consistent with the proposal that Mdm2 functions to regulate p53 activity in the cell. To examine whether the developmental defect in Mdm2-null mice results from alterations in Mdm2-mediated gene expression, or is due to a loss in the ability of Mdm2 to regulate p53 activity, *mdm2*^{tm}/+ and *p53* −/− mice[11] were intercrossed to produce mice which were *mdm2*^{tm}/+, *p53* +/−. These compound heterozygous mice were mated and the resulting progeny genotyped for *Mdm2* and *p53* status by Southern analysis (Fig. 3*b*). Nine viable *mdm2*^{tm}/*mdm2*^{tm} mice were obtained from this cross, all of which were

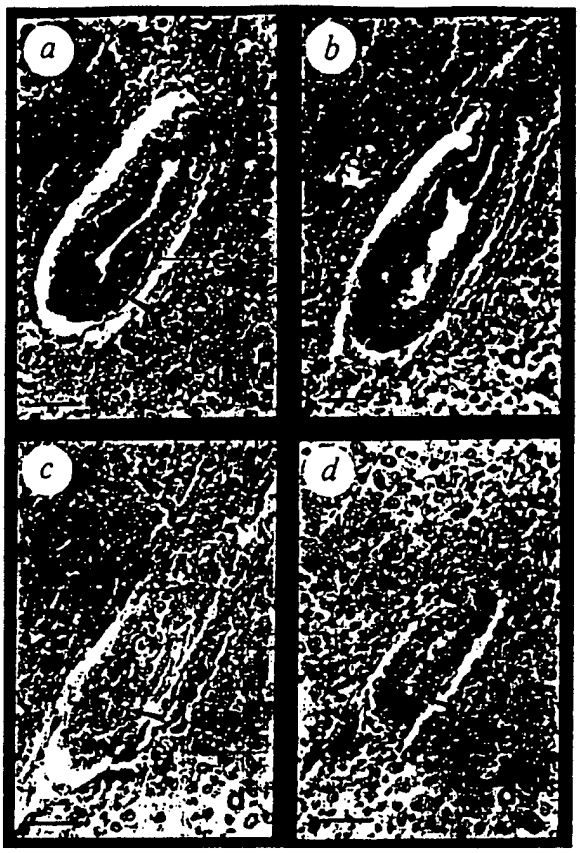

FIG. 2 Histology of embryos from *mdm2*^{tm}/+ intercrosses on day 6.5 of gestation. *a*, *b*, Sagittal sections of phenotypic wild-type embryos exhibiting well-organized embryonic ectoderm (arrows). *c*, *d*, Sagittal sections of mutant embryos. The embryos contain fewer embryonic ectoderm cells, lack a normal wild-type architecture, and are smaller in size than the wild-type embryos. Abbreviations: d, maternal decidua; ec, ectoplacental cone; e, endoderm. Scale bar, 50 μm.
METHODS. Embryos were collected from female partners of *mdm2*^{tm}/+ intercrosses on day 6.5 post-coitum and fixed in Bouin's solution. Embedding, sectioning and staining of the embryo sections with haematoxylin and eosin were performed as described previously[23].

p53−/−. In addition, matings between *mdm2*^{tm}/+, *p53* +/− and *mdm2*^{tm}/+, *p53* −/− mice have yielded 7 *mdm2*^{tm}/*mdm2*^{tm} mice, all of which were *p53*−/−. The *mdm2*^{tm}/*mdm2*^{tm}, *p53* −/− mice were recovered at the expected ratios in the two crosses, accounting for the non-viability of the *mdm2*^{tm}/*mdm2*^{tm} embryos on the *p53* +/+ and *p53* +/− background. Furthermore, 17 double-null mice have been recovered from *mdm2*^{tm}/*mdm2*^{tm}, *p53* −/− intercrosses. Thus absence of p53 rescues the embryonic lethality seen in Mdm2-deficient mice. These results indicate that Mdm2 functions in development primarily as a regulator of p53 activity. Although these results do not exclude the possibility that Mdm2 possesses other functions, these functions cannot be critical in development because Mdm2/p53 double-null mice are developmentally normal, fertile and appear phenotypically indistinguishable from wild-type mice.

The mutation created in *Mdm2* does not alter the 5′ portion of the gene which encodes the Mdm2 domain that binds with p53 (ref. 12). It is conceivable that a truncated Mdm2 protein might be produced from the targeted allele that could bind with p53 and induce lethality in mice. This lethality would then be rescued in mice nullizygous for p53. To test this possibility, RNA

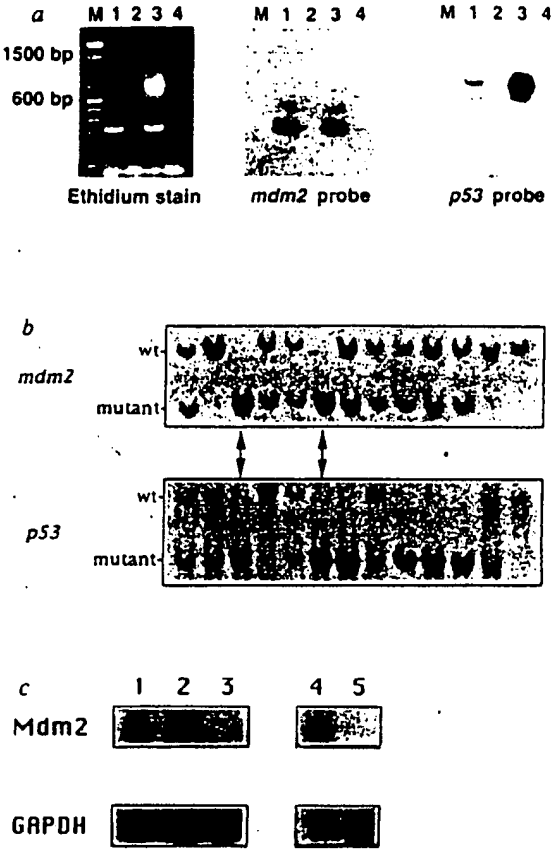

FIG. 3 *a*, Reverse transcription–PCR amplification (RT–PCR) of RNA from day 6.0–6.5 wild-type embryos. Oligonucleotide primers for both *Mdm2* and *p53* cDNA were present in the PCR reaction. The larger (840 bp) and smaller (344 bp) fragments observed after staining of the gel with ethidium bromide (left) are amplification products of *p53* and *Mdm2* cDNA, respectively. Lane 1, RT–PCR products of day 6.0–6.5 embryo RNA. Lane 2, as lane 1 except no reverse transcriptase was present in the RT–PCR reaction. Lane 3, RT–PCR products of wild-type primary embryo fibroblast RNA. Lane 4, PCR reaction performed in the absence of template. Lane M is a 100-bp DNA size standard. Southern analysis of the PCR products using either an *Mdm2*-specific oligonucleotide probe (middle) or a *p53*-specific oligonucleotide probe (right) was performed to confirm the identity of the PCR fragments. *b*, Southern analysis of progeny of $mdm2^{tm1}/+$, *p53* +/− intercrosses. Compound heterozygous mice were mated and the offspring genotyped for *Mdm2* (top) and *p53* (bottom) by Southern analysis. Two of the mice were determined to be $mdm2^{tm1}/mdm2^{tm1}$. These two mice were also *p53* −/− (double-headed arrows). *c*, Northern analysis of total RNA isolated from the kidney and/or spleen of mice which were either wild-type (lane 1), *p53* −/− (lanes 2 and 4), or $mdm2^{tm1}/mdm2^{tm1}$, *p53* −/− (lanes 3 and 5). Top, *Mdm2* cDNA probe; bottom, *GAPDH* probe, used as a control for load and integrity of RNA. No *Mdm2* transcript is detected in the Mdm2, p53- double-null tissue.

METHODS. Total RNA was isolated from primary embryo fibroblasts for RT–PCR and from whole tissue for either RT–PCR or northern analysis using RNAzol B as described by the manufacturer (Tel-Test, Friendswood, TX). RT–PCR was performed using the Superscript Preamplification System (Gibco-BRL, Bethesda, MD) followed by a 40-cycle PCR using *Mdm2* primers (5'-ATGTGCAATACCAACATCTCTGTGTC-3' and 5'-GCTGACTTACAGCCACTAAATTTC-3') and *p53* primers (5'-CTGCAGTCTGGGACAGCCAAGTC-3' and 5'-GTCAGTCAGACTCAGTCCGGGGTG-3'). Of each sample, 40% was visualized on a 2% agarose-ethidium bromide gel. Southern analysis was performed on the PCR products using an oligonucleotide probe which hybridizes to either *Mdm2* or *p53* coding sequences which are internal to the primers used in the PCR reaction. Southern analysis was performed on genomic DNA isolated from tail biopsies of four-week-old mice. The DNA was digested with *Eco*RI, and the Southern blot probed with the *Mdm2* SacI–ScaI 3' probe described in Fig. 1. After autoradiography, the blot was stripped and reprobed with a *p53* exon 11 probe that recognizes a 16-kb *Eco*RI fragment in wild-type DNA, and an 8-kb *Eco*RI fragment in the mutated *p53* gene[12]. The double-headed arrows indicate genomic DNA of mice which are both Mdm2- and p53-null.

was isolated from the kidney and spleen of mice which were either wild type, p53-deficient, or p53- and Mdm2-deficient. Northern analysis failed to detect any *Mdm2* transcripts in $mdm2^{tm1}/mdm2^{tm1}$ tissue (Fig. 3*c*). Furthermore, $mdm2^{tm1}/+$ mice that are wildtype for p53 are viable. Thus it is unlikely that a hypothetical truncated Mdm2–p53 complex causes the embryonic lethality seen in $mdm2^{tm1}/mdm2^{tm1}$ mice.

The p53 protein has been shown to regulate cell growth negatively by arresting cells late in the G1 phase of the cell cycle[13–17]. In resting cells stimulated with serum, the levels of Mdm2 and Mdm2–p53 complex rise late in G1, suggesting that Mdm2 can downregulate the ability of p53 to block progression of the cell cycle[18]. More recently, overexpression of exogenous *Mdm2* in transfected cells has been found to inhibit the ability of these cells to undergo p53-mediated growth arrest following treatment with ionizing radiation, providing direct evidence that Mdm2 is involved in inhibiting p53 function in a known pathway[19]. Our data indicate that Mdm2 plays an important role in development by inhibiting p53 function during a period in gestation when the mean cell cycling time of primitive ectoderm decreases dramatically in normal embryos[9]. Thus the developmental defect in Mdm2-null mice might arise as a result of failure to inhibit p53-mediated suppression of cell cycling during this time of rapid cell division. This finding might also explain the difficulty experienced by researchers attempting to construct transgenic mice which overexpress wild-type *p53* (A. Bernstein and J. Butel, personal communication). □

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Rescue of early embryonic lethality in *mdm2*-deficient mice by deletion of *p53*

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THE gene *p53* encodes a transcriptional activator[1,2] of genes involved in growth arrest[3,4], DNA repair[5] and apoptosis[6–8]. Loss of *p53* function contributes to tumour development *in vivo*[9–11]. The transcriptional activation function of p53 is inactivated by interaction with the *mdm2* gene product[12–14]. Amplification of *mdm2* has been observed in 36% of human sarcomas, indicating that it may represent an alternative mechanism of preventing p53 function in tumour development[15]. To study *mdm2* function *in vivo*, we generated an *mdm2* null allele by homologous recombination. *Mdm2* null mice are not viable, and further analysis revealed embryonic lethality around implantation. To examine the importance of the interaction of MDM2 with p53 *in vivo*, we crossed mice heterozygous for *mdm2* and *p53* and obtained progeny homozygous for both *p53* and *mdm2* null alleles. Rescue of the *mdm2*$^{-/-}$ lethality in a *p53* null background suggests that a critical *in vivo* function of MDM2 is the negative regulation of p53 activity.

The *mdm2* gene was cloned from a murine 129 library and characterized (Fig. 1). To mutate the *mdm2* gene in mouse embryonic stem (ES) cells, we generated a vector that deletes two-thirds of the MDM2 protein-coding sequences, including the acidic and zing-finger-like domains[16] (amino acids 221–489) by replacing them with a neomycin-resistance expression cassette (Fig. 1*a*, *b*). Correctly targeted clones were detected by the presence of an additional 5.2-kb band using either 5′ or 3′ probes external to the region of vector homology (Fig. 1*c*). One correctly targeted ES clone successfully contributed to the germ line of chimaeric mice generated by blastocyst injection.

Mice heterozygous for the *mdm2* deletion allele appeared phenotypically normal and were fertile. However, no homozygous mutants were identified among more than 50 offspring born from matings between heterozygotes, indicating that *mdm2* mutant mice died during embryogenesis. Therefore, embryos from timed matings between heterozygotes were analysed at different stages of gestation (Table 1*a*). Empty deciduae were commonly

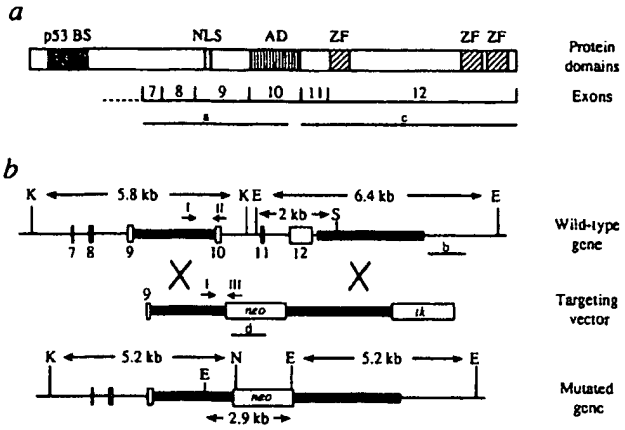

FIG. 1 Disruption of the *mdm2* gene. *a*, The functional domains of the MDM2 protein and exons 7–12 encoding those domains. *b*, Partial exon/intron structure of the *mdm2* gene, the targeting vector, and predicted homologous recombination events. c, Southern blot analysis of genomic DNA isolated from the ES cell clone that resulted in germline transmission; and offspring from heterozygous matings. DNA from ES cell clones was digested with *Kpn*I and *Not*I and hybridized with probe a (left) or digested with *Eco*RI and hybridized with probe b (middle). DNA from progeny was digested with *Eco*RI and hybridized with probes c and d (right). Abbreviations: p53 BS, p53 binding site; NLS, nuclear localization signal; AD, acidic domain; ZF, zinc-finger; K, *Kpn*I; N, *Not*I; E, *Eco*RI; S, *Stu*I; kb, kilobases; W, wild type; *T*, targeted. Roman numerals denote primers used in PCR amplification. Lines identified by lowercase letters indicate probes used in Southern analysis.
METHODS. A 129/SvEv mouse genomic library was screened with a PCR-amplified *mdm2* cDNA probe. Three overlapping phage clones were obtained that spanned the entire *mdm2* gene consisting of 12 exons dispersed in approximately 20 kb of DNA as determined by Southern analysis, PCR amplification and sequencing (R.M.L., unpublished observations). A PGKneobpA resistance expression cassette was inserted in reverse orientation relative to the direction of *mdm2* transcription[25]. A MC1tkpA herpes simplex virus thymidine kinase expression cassette was added to the long arm of homology to enrich for homologous recombinants using negative selection with 1-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)-s-iodouracil (FIAU)[26]. Linearized vector (25 μg) was electroporated into 10^7 AB-1 ES cells that were subsequently cultured in the presence of G418 and FIAU[25,27]. When the vector is recombined with the endogenous *mdm2* gene, new *Not*I and *Eco*RI sites are introduced. G418/FIAU-resistant ES clones (1000) were initially screened by Southern blot analysis using probe a. Correctly targeted clones were expanded for further Southern blot analysis using probe b. Six correctly targeted ES clones were identified. Three of the *mdm2* mutant ES clones were microinjected into C57BL/6J blastocysts and transferred to the uterine horn of day 2.5 pseudopregnant females. Resulting chimaeras were bred to C57BL/6J mice. One of three mutant ES cell lines injected contributed to the germ line of mice.

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TABLE 1 Genetic analysis of mutants

(a) *mdm2* mutant embryos

Stage	Phenotypes				Genotypes		
	$mdm2^{+/-} \times mdm2^{+/+}$		$mdm2^{+/-} \times mdm2^{+/-}$		$mdm2^{+/+}$	$mdm2^{+/-}$	$mdm2^{-/-}$
	Normal	Abnormal*	Normal	Abnormal			
E10.5	19	0	6	4	2	4	0
E 8.5	30	1	38	7	4	17	0
E 7.5	33	2	49	14	7	9	0
E 6.5	9	0	11	5	1	4	0
Total	91	3	104	30	14	34	0

(b) *mdm2*/*p53* mutant mice

Genotype		No. of mice
$p53^{+/-}$ $mdm2^{+/-}$	$\times p53^{+/-}$ $mdm2^{+/-}$	
$p53^{+/-}$	$mdm2^{+/+}$	3
$p53^{+/-}$	$mdm2^{+/-}$	5
$p53^{+/-}$	*mdm2*	0
$p53^{+/-}$	$mdm2^{-/-}$	7
$p53^{+/-}$	$mdm2^{+}$	11
$p53^{+/-}$	*mdm2*	0
p53	$mdm2^{+/+}$	1
p53	$mdm2^{+/-}$	7
p53	*mdm2*	2

Genotype		No. of mice
p53 mdm2	$\times p53^{+/-}$ $mdm2^{+/-}$	
$p53^{+/-}$	$mdm2^{-/-}$	12
$p53^{+/-}$	*mdm2*	0
p53	$mdm2^{+/-}$	9
p53	*mdm2*	9

a, Genotypic analysis of *mdm2* in embryos generated from heterozygous crosses. E10.5 embryos were analysed by Southern blots using probes c and d (Fig. 1). The remaining embryos were genotyped by polymerase chain reaction (PCR) using a three-primer combination: I, 5'-TGTGGCTGGAGCATGGGTATTG-3'; II, 5'-ATCTGAGAGCTCGTGCCCTTCG-3'; III, 5'-GGCGGAAAGAACCAGCTGGGGC-3'. I and II amplify the wild-type *mdm2* allele (415 bp), and I and III amplify the mutant allele (284 bp), as is shown in Fig. 3a, *b*. *b*, Analysis of *mdm2* and *p53* genotypes in offspring generated from *mdm2*/*p53* crosses as indicated. The genotypes were determined as shown in Fig. 3.

* The abnormal phenotype is defined as an empty decidua.

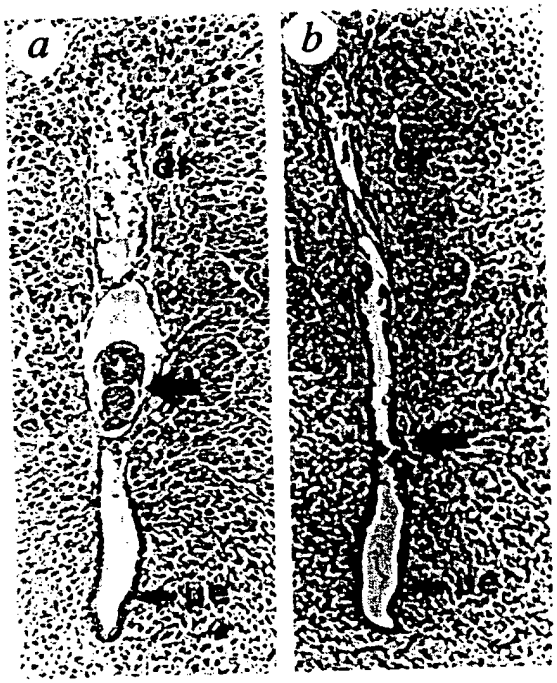

FIG. 2 Histological analysis of E5.5 embryos from a cross between two *mdm2* heterozygous mutant mice. *a*, Transverse section through the decidua of a normal embryo. *b*, Transverse section through a decidua of an abnormal E5.5 embryo. Large arrows mark the location or the predicted location of the embryo. Abbreviations: ue, uterine epithelium; dr, decidual reaction.

METHODS. Embryos were processed for histological analysis by fixation in Bouin's fixative, dehydrated and embedded in paraffin. Sections 6 μm thick were cut and stained with haematoxylin and eosin.

seen in these crosses: of a total of 134 deciduae analysed, 30 (22%) were empty. In contrast, in crosses between *mdm2* heterozygotes and normal mice, this abnormality was seen in only 3% of the deciduae analysed. We did not find any *mdm2* homozygous mutant embryos from days E6.5–E10.5 (Table 1*a*). Blastocysts were then isolated from a heterozygous *mdm2* mutant female that had been mated with a heterozygous *mdm2* mutant male. One of the eight blastocysts examined contained no wild-type *mdm2*. Our identification of a viable *mdm2* null blastocyst and the lack of *mdm2* null embryos indicates that the time of death must be between implantation and E6.5.

To examine the phenotype in more detail, we isolated, fixed and paraffin embedded the deciduae of an $mdm2^{+/-}$ female crossed with an $mdm2^{+/-}$ male 5.5 days post-coitum. The embryos were sectioned and, although they could not be genetically typed, the two phenotypes were obvious, one normal and the other abnormal (Fig. 2). The abnormal embryo had few, if any, cells (Fig. 2*b*, large arrow) compared with a normal embryo (Fig. 2*a*, large arrow), but had formed an active site of implantation, visible by the decidual reaction, thus explaining the presence of empty deciduae at later stages of development.

One of the functions of MDM2 identified in tissue-culture experiments is the negative regulation of p53 growth suppression[17] and transcriptional activation[12-14]. Indeed, *p53*, *p21* and *mdm2* are highly expressed in ES cells as compared with normal tissues by northern analysis, which indicates expression early in development (data not shown). We therefore hypothesized that the embryonic lethality seen in *mdm2* homozygous mutants was due to an inability to downregulate p53 function which could lead to growth arrest or apoptosis. Because a *p53* null mouse is viable[10,11], we tested our hypothesis by interbreeding mice heterozygous for both *mdm2* and *p53* genes. Striking genotyping of offspring revealed the presence of *mdm2* homozygous mutants in a *p53* null background (Fig. 3*a*, *b*). From the mendelian ratio, one of 16 mice from this cross could be expected to be a double homozygote. Of 36 progeny born from matings of double heterozygote crosses, two were homozygous for the *mdm2 and p53* mutant genes (Table 1*b*). In addition, no homozygous mutant *mdm2* mice were identified in either a wild-

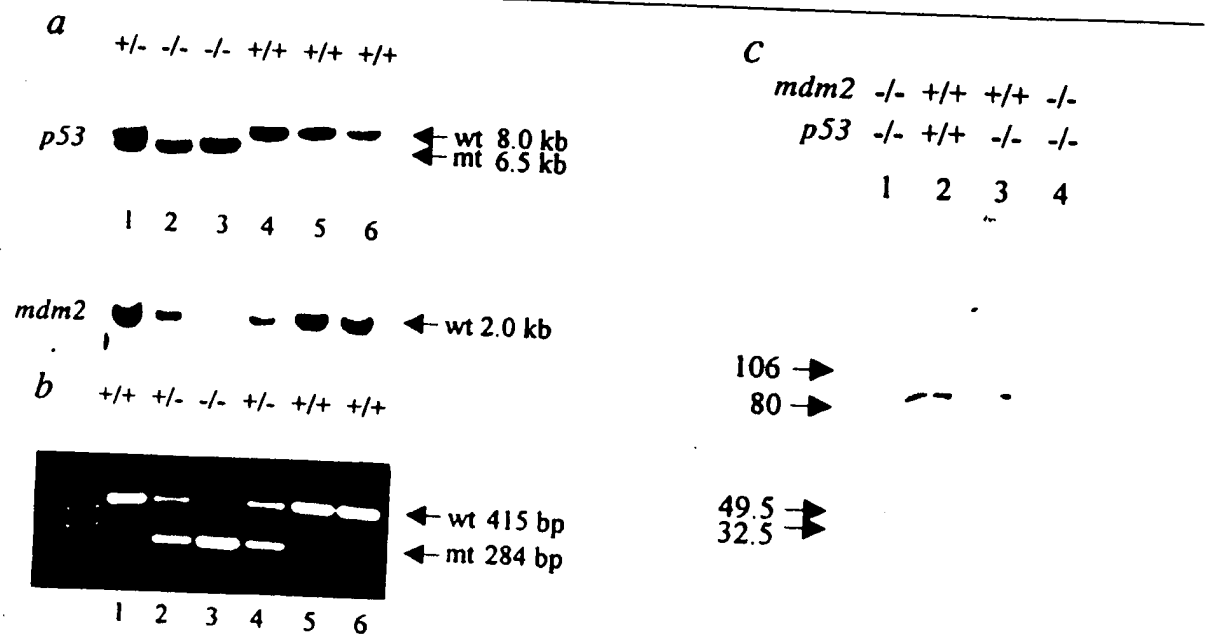

FIG. 3 *a* and *b*, Southern blot and PCR analysis of progeny from matings between mice heterozygous at both *mdm2* and *p53* alleles. The mice heterozygous for the *mdm2* mutation created in this study were mated with p53 null mice (Jackson laboratory)[11]. *a*, The progeny heterozygous at both genes were mated, and their progeny analysed for the homozygous deletion of *mdm2* by Southern blot using probe c (Fig. 1). The same blots were analysed for the *p53* null allele using a probe as described[11]. *b*, The *mdm2* genotypes of the mice were also checked by PCR amplification using the primers described in Table 1*a*. *c*, Western blot analysis of *mdm2* mutants. Total protein was extracted from the uteri of $p53^{-/-}$ $mdm2^{-/-}$ (lanes 1 and 4), $p53^{+/+}$ $mdm2^{+/+}$ (lane 2), and $p53^{-/-}$ $mdm2^{+/+}$ mice (lane 3). We chose the uterus because it contains high levels of MDM2 (S. de Rozieres and G.L., unpublished observations). The position of *M*, markers is shown on the left.

METHODS. For the western blot, total protein extracted (50 μg) from the uteri of mice was resolved by electrophoresis through an SDS–7.5% polyacrylamide gel. Low-range prestained *M*, standards were also used (Bio-Rad). Blots were performed using an MDM2 antibody prepared as follows: the *mdm2* cDNA was cloned into pRSETB (Invitrogen), which added a His tag hexamer. MDM2 was purified by immobilized metal affinity chromatography and used as an antigen in rabbits. The blots were developed using horseradish peroxidase as the secondary antibody and the ECL method (Amersham). Fast green staining of the blots confirmed that equivalent amounts of protein were present in each lane.

heterozygous *p53* background, indicating that heterozygosity of *p53* is insufficient to rescue the embryonic lethality of *mdm2* nulls. To further substantiate this finding, males that were homozygous mutant at both loci were mated with females that were heterozygous at both loci. Of viable mice born, 30% had homozygous deletions in both genes (Table 1*b*). The ratio is slightly viewed because one-quarter of the animals are expected to be homozygous for *mdm2* and heterozygous for *p53* and should therefore be expected to die *in utero*.

To show that an abnormal MDM2 polypeptide was not generated, we performed western blot analysis of tissues from *mdm2/p53* mutant homozygous mice. Although wild-type *mdm2* mice, regardless of their *p53* status, contained a protein of the expected relative molecular mass (*M*, 90K), the *mdm2/p53* null mutant mice did not make the p90 MDM2 polypeptide (Fig. 3*c*). Exposure of several western blots for longer times did not reveal the presence of a small peptide, although its existence cannot be completely ruled out.

Our *in vivo* analyses demonstrated a tight functional relationship between p53 and MDM2 in early development. Our first important observation was that the loss of *mdm2* resulted in early embryonic lethality. The embryos died between implantation and 5.5 days of development. Within eight cell divisions, approximate number of divisions achieved by 5.5 days of development, the null embryo was no longer recognizable as a gene affecting proliferation. Because MDM2 is a negative regulator of p53 function[12–14], we suspect that p53 function was inactivated in our embryos. Thus, although loss of *p53* caused no problems for the embryo, the absence of its negative regulator was lethal. Because stabilization of p53 leads to growth arrest or apoptosis after DNA damage[5,7,18,22], it is possible that either of these mechanisms might cause embryonic death in the *mdm2* null mice. The early lethality of the $mdm2^{-/-}$ mice precludes the observation of any later effects. Further, our results emphasize the importance of assaying p53 functionally, as the physiological ratio of p53 to MDM2 will influence p53 activity.

The second major observation is that the *mdm2* null phenotype was rescued by deletion of *p53*: double-homozygous-mutant mice were viable. Subtle abnormalities may exist, however, as the $mdm2^{-/-}$ $p53^{-/-}$ and $mdm2^{+/-}$ $p53^{-/-}$ females had few litters and very few pups in their litters. These data imply a function for MDM2 in addition to inactivation of p53. Indeed, interactions between MDM2 and the retinoblastoma protein have recently been identified[23]. Furthermore, interaction of MDM2 with E2F1 and DP1 augments their transcriptional activation of genes involved in S-phase progression[24].

Our results provide genetic evidence for the interaction of MDM2 and p53 *in vivo*, and also indicate that the *mdm2* null embryos die because p53 function cannot be downregulated. Investigation of viable double-null homozygotes might allow us to determine whether the loss of *mdm2* in mice affects the timing and range of tumour development in the *p53* null background. □

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Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53

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THE *Mdm2* proto-oncogene was originally identified as one of several genes contained on a mouse double minute chromosome present in a transformed derivative of 3T3 cells[1]. Overexpression of *Mdm2* can immortalize primary cultures of rodent fibroblasts[2]. Human *MDM2* is amplified in 30–40% of sarcomas, and is overexpressed in leukaemic cells[3,4]. The Mdm2 oncoprotein forms a complex with the p53 tumour-suppressor protein and inhibits p53-mediated transregulation of gene expression[5,6]. Because *Mdm2* expression increases in response to p53, Mdm2–p53 binding may autoregulate *Mdm2* expression and modulate the activity of p53 in the cell[7,8]. We have created Mdm2-null and Mdm2/p53-null mice to determine whether Mdm2 possesses developmental functions in addition to the ability to complex with p53, and to investigate the biological role of Mdm2–p53 complex formation in development. Mice deficient for Mdm2 die early in development. In contrast, mice deficient for both Mdm2 and p53 develop normally and are viable. These results suggest that a critical role of Mdm2 in development is the regulation of p53 function.

Mdm2 was cloned from a 129-strain genomic library and the gene structure was characterized to allow the creation of a replacement vector for gene targeting in embryonic stem (ES) cells (Fig. 1*a*). Positive and negative selection of transfected AB2.1 ES cells led to the isolation of one clone which had replaced the 7.1-kilobase (kb) region of *Mdm2* which encodes all putative transcription function motifs of Mdm2 with an *hprt* reporter gene. Injection of the targeted ES cells into host blastocysts gave rise to chimaeric mice which transmitted the mutated *Mdm2* allele (*mdm2^{tm1}*) to F_1 progeny (Fig. 1*b*). Intercrosses between mice heterozygous for the targeted allele (*mdm2^{tm1}*/+) were performed and the offspring genotyped by Southern ana-

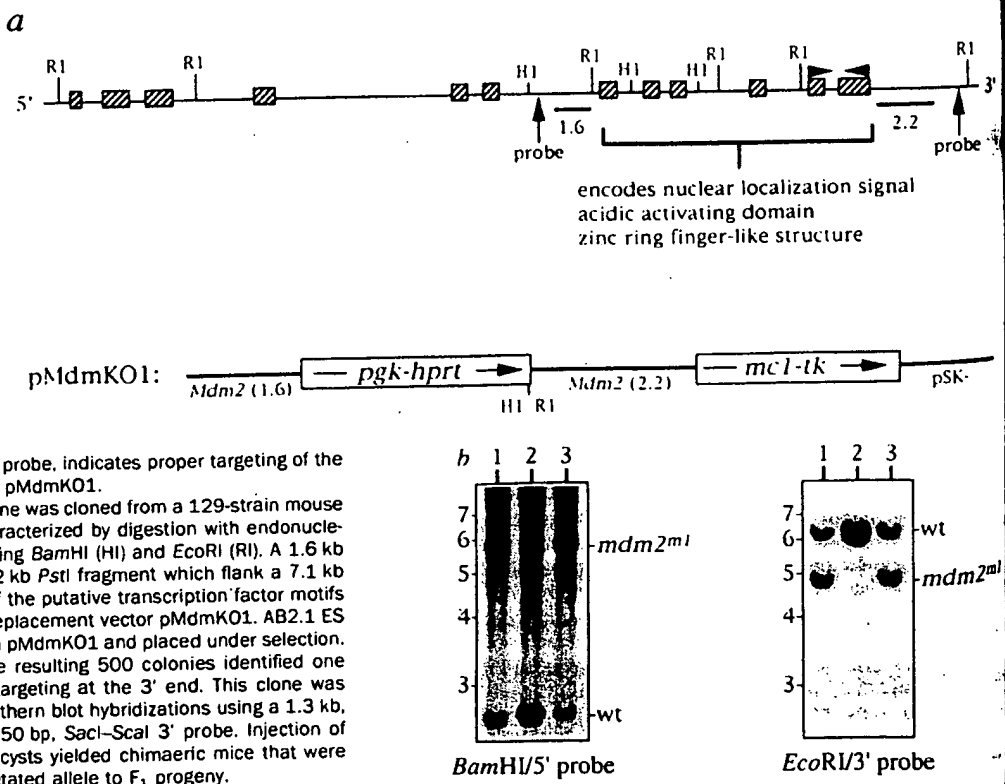

FIG. 1 Gene targeting in *Mdm2*. *a*, Structure of *Mdm2* gene and targeting vector. A replacement targeting vector containing 1.6 kb of 5' homology and 2.2 kb of 3' homology was constructed with markers for positive selection (hprt mini-gene)[20] and negative selection (thymidine kinase)[21]. *b*, Southern analysis of targeted ES cell DNA (lane 1), and genomic DNA from a wild-type F_1 progeny mouse (lane 2) and mutant F_1 progeny mouse (lane 3). The presence of a 5.7 kb *Bam*HI band following hybridization with a 5' probe, and the presence of a 4.7 kb *Eco*RI band following hybridization with a 3' probe, indicates proper targeting of the *Mdm2* gene in the ES clone by pMdmKO1.

METHODS. The entire *Mdm2* gene was cloned from a 129-strain mouse genomic phage library and characterized by digestion with endonuclease restriction enzymes, including *Bam*HI (HI) and *Eco*RI (RI). A 1.6 kb *Kpn*I–*Eco*RI fragment and a 2.2 kb *Pst*I fragment which flank a 7.1 kb region of *Mdm2* encoding all of the putative transcription factor motifs were used to create the gene replacement vector pMdmKO1. AB2.1 ES cells[20] were electroporated with pMdmKO1 and placed under selection. Mini-Southern analysis[22] of the resulting 500 colonies identified one clone which exhibited proper targeting at the 3' end. This clone was expanded and analysed by Southern blot hybridizations using a 1.3 kb, *Bam*HI–*Kpn*I 5' probe and a 350 bp, *Sac*I–*Sca*I 3' probe. Injection of these ES cells into host blastocysts yielded chimaeric mice that were capable of transmitting the mutated allele to F_1 progeny.